BEHAVIORAL AND COGNITIVE IMPAIRMENTS ACROSS THE LIFE SPAN

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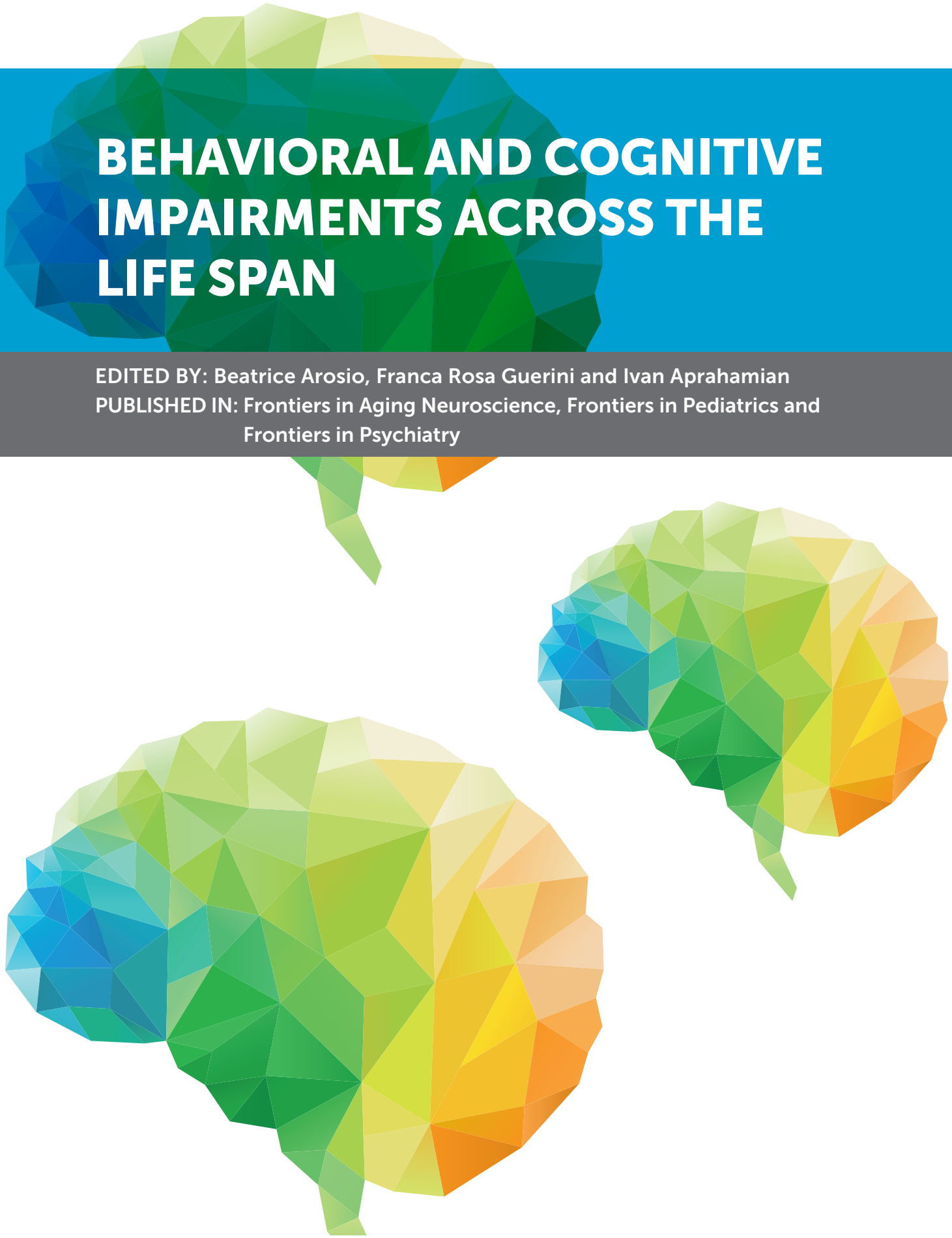

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BEHAVIORAL AND COGNITIVE IMPAIRMENTS ACROSS THE LIFE SPAN

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Dietary Diversity Is Associated With Memory Status in Chinese Adults: A Prospective Study

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Background and Aim: Subjective memory complaints are common in elderly people. Nutrition plays an important role in keeping brain health, however, the evidence on dietary diversity and subjective memory status is limited. This study aimed to investigate the effect of dietary diversity score (DDS) on memory status in Chinese adults in a prospective cohort study.

Methods: Data of the China Health and Nutrition Survey was used in this study. A total of 4356 participants aged 50 years or older were enrolled in the analysis. DDS was calculated based on the dietary recall data collected in the wave of 2011. Information on self-report memory status (OK, good, or bad) and memory change in the past 12 months (stayed the same, improved, or deteriorated) were obtained from the wave of 2015. A memory score was calculated based on a subset of items of the Telephone Interview for Cognitive Status-modified. Multinomial logistic regression models were used to estimate the associations of DDS with memory status and memory change, and linear regression models were carried out to estimate the association between DDS and memory score.

Results: In the study population, the percentages of participants who thought their memory was OK, bad, and good were 43.3, 24.3, and 32.4%, respectively. There were 1.4% of participants reported memory improvement in the past 12 months and 47.2% reported memory decline. Average memory score among participants was 12.8 ± 6.1. Compared with participants who thought their memory was OK, a higher DDS was associated with self-reported good memory (Odds Ratio [OR] 1.15, 95%CI 1.07–1.24) and inversely associated with bad memory (OR 0.82, 95%CI 0.75–0.89). In subgroup analysis, however, in participants aged 65 years and above, the association between DDS and self-reported good memory was insignificant (OR 1.09, 95%CI 0.94–1.25). Compared with participants whose memory stayed the same, higher DDS was inversely

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associated with memory decline (OR 0.85, 95%CI 0.80–0.91). Besides, higher DDS was associated with higher memory score (β 0.74, 95%CI 0.56–0.91).

Conclusion: This study revealed that higher DDS was associated with better memory status and was inversely associated with self-reported memory decline in Chinese adults.

Keywords: memory status, memory decline, dietary diversity, adults, prospective study

INTRODUCTION

Subjective memory complaints are common in elderly people (Caramelli and Beato, 2008), which affects the quality of life of elderly people negatively. Memory complaint is not only an age-related phenomenon but also an early signal of Alzheimer's disease and dementia (Jonker et al., 2000; Peters et al., 2019). In community-dwelling elderly individuals, there is a 25–50% prevalence rate of memory complaints (Jonker et al., 2000). In 2015, people over 60 years constituted 12% of the world's population, and the population is aging at a faster pace than the past (World Health Organization [WHO], 2018). Memory loss has become an important public health issue and a social concern (Centers for Disease Control and Prevention, 2019; World Health Organization [WHO], 2019), thus actions to prevent early memory decline will be beneficial to both the quality of individual life and the burdens of society.

Lifestyle intervention as a cost-effective way to prevent some age-related health diseases has been recognized by increasingly more people (Katz et al., 2018). Healthy diets can delay dementia progression and reduce the risk of Alzheimer's disease in the elderly (George and Reddy, 2019). A cohort study in Australia showed high consumption of fruit, vegetables, and protein-rich food was associated with lower odds of self-reported memory loss (Xu et al., 2020). Another study in Chinese showed higher fish consumption was associated with a slower decline in memory in adults aged 65 years and above (Qin et al., 2014). Besides, a cohort study revealed that higher adherence to the Mediterranean diet was inversely associated with poor subjective cognitive function (Bhushan et al., 2018). Several studies showed that high dietary diversity decreased the risk of cognitive decline (Clausen et al., 2005; Otsuka et al., 2017). However, evidence on dietary diversity and memory status from large-scale, prospective studies were limited.

Dietary diversity has long been recognized as a key element of diet. As a tool to assess both nutrient adequacy of individual and food security of household (Kennedy et al., 2011; Salehi-Abargouei et al., 2016), dietary diversity score (DDS) has been widely used in different populations (Salehi-Abargouei et al., 2016). Several age-related diseases, including diabetes (Conklin et al., 2016) and hypertension (Kapoor et al., 2018), are reported to be inversely associated with DDS. As the elderly people usually experience accelerated mobility decreasing, degeneration of the digestive system, and decline in appetite (Favaro-Moreira et al., 2016; Zhao et al., 2019), they may face higher risks of nutrient deficiency (Ahmed and Haboubi, 2010), which may further generate negative effects on memory. In this study, we investigated the effect of dietary diversity on memory status in Chinese adults aged 50 years and above.

MATERIALS AND METHODS

Study Population

Data was obtained from the China Health and Nutrition Survey (CHNS). The CHNS is a national-wide, dynamic cohort study initialed in 1989, aiming to understand the health and nutrition status of Chinese and how they are affected by social and economic transformation. Details about the CHNS has already been published (Zhang et al., 2014). Data collected in the wave of 2011 and 2015 were used in this study to estimate the dietary diversity and to obtain information on memory status respectively. The inclusion criteria of our study included aged 50 years and above in the wave of 2011, participated in the dietary survey in the wave of 2011, participated in the follow-up survey in wave 2015. The exclusion criteria included diagnosed with apoplexy, uncertain about one's memory status, and having missing values on covariates. In the end, 4356 participants from 12 areas of China (Beijing, Liaoning, Heilongjiang, Shanxi, Jiangsu, Shandong, Henan, Hubei, Hunan, Guangxi, Guizhou, and Chongqing) were included in our analyses (**Figure 1**).

Dietary Survey and Dietary Diversity Score

Dietary intake was assessed by individual dietary recall for 3 consecutive days in combination with family food weight inventory. Participants were asked to report all the foods and beverages they consumed during a 24-h period. More details about the dietary survey process have been described elsewhere (Zhai et al., 1996). The present study used dietary data collected in the wave of 2011. All food items were divided into eight food categories (cereals and tubers, vegetables, fruits, meat, soybeans and nuts, eggs, aquatic products, and milk and dairy products). If one participant consumed any food from a certain food category in the past 24 h, then he would get one point for that food category, with a total score of eight. Average daily scores were calculated for each participant. Besides, participants' daily energy and nutrient intakes were estimated based on the China food composition databases (Yang, 2005; Yang et al., 2009).

Ascertain of Memory Status

In the wave of 2015, participants' memory status and memory change in the past 12 months were surveyed. For memory status,

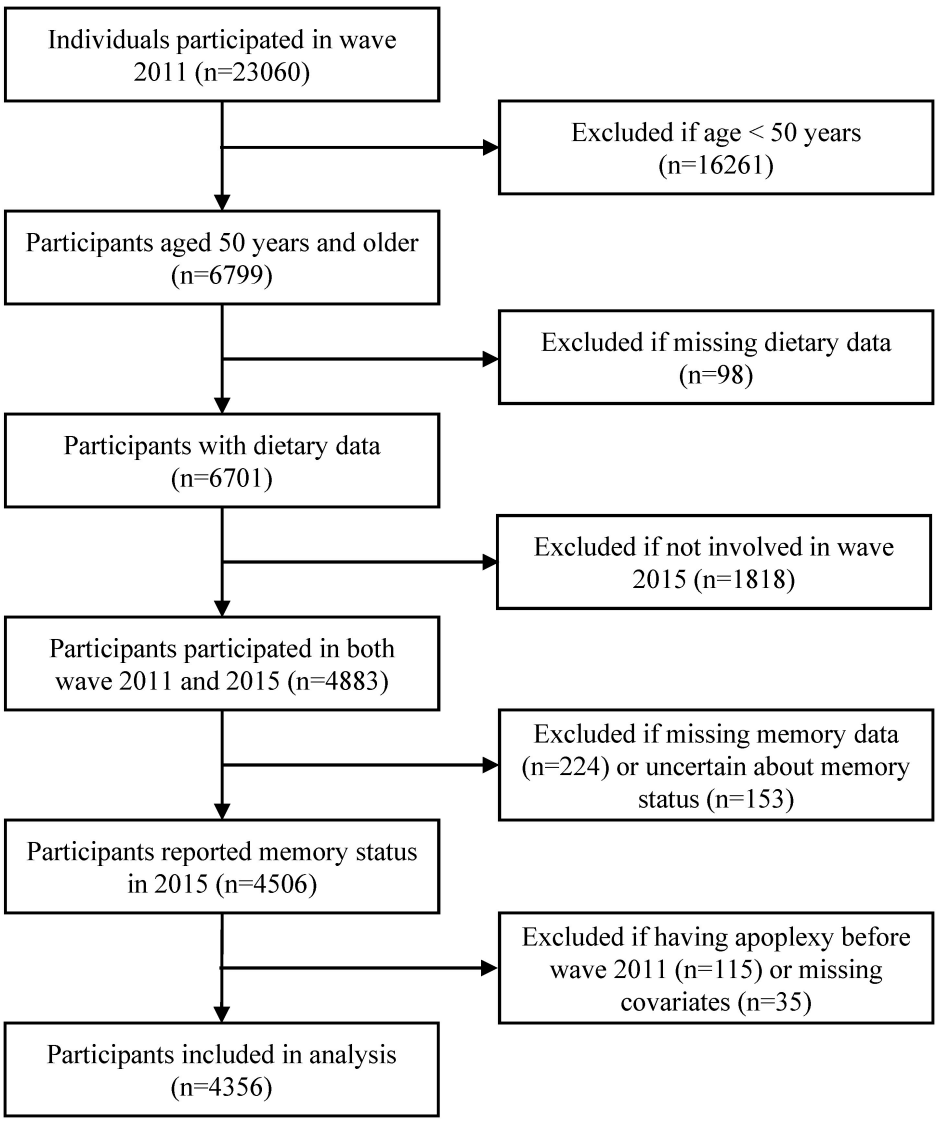

FIGURE 1 | Flow chart of sample selection.

participants were asked "How is your memory?" (very good, good, OK, bad, very bad, or unknown). We classified participants' memory status into good (very good/good), OK, and bad (very bad/bad) for further analysis. For memory change over last year, participants were asked "In the past 12 months, how has your memory changed?" (improved, stayed the same, deteriorated, or unknown). Individuals who were unknown about their memory status or memory change were excluded from the analysis.

Besides, a subset from the Telephone Interview for Cognitive Status-modified (Brandt et al., 1988; van den Berg et al., 2012) was used to determine participants' memory function. The test items included immediate and delayed free-recall test (ten words) and the Serial 7s test. These tests assess participants' verbal memory and working memory (van den Berg et al., 2012). Details about the tests were published elsewhere (Herzog and Wallace, 1997; Qin et al., 2014). Participants whose answer was "unknown" did not get a score for the test item. Total memory scores rank from 0 to 25. 98.6% of participants took this test.

Covariates

Covariates were obtained from the wave of 2011. Covariates of sociodemographic characteristics and lifestyle behaviors used in this study included age (continuous), gender (men/women), living region (southern/northern China), education level (primary school or lower, lower middle school, or middle school or above), alcohol consumption (never drink, <3, or ≥3 times a week), smoking status (never smoke, used to smoke, currently smoke), and annual per capita household income. 21.9% of participants had missing data on income information, and missing values of annual per capita household income were replaced by the medians of each survey site. Income was classified into as low, middle, or high, corresponding to annual

per capita household income 10,000 and below, 10,000–20,000, and over 20,000 RMB, respectively. Besides, medical history used in this analysis included previously diagnosed apoplexy (yes/no), diabetes (yes/no), myocardial infarction (yes/no), and hypertension (yes/no). Since blood pressures were measure in the wave of 2011 (99.5% of participants took this test), participants who had been diagnosed with hypertension or whose systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$90 mmHg were all regarded as hypertensive patients (Hua et al., 2019).

Statistics

Normally distributed continuous variables were presented with Means and SDs; otherwise, medians and quartiles were used. Categorical variables were presented with percentages. Differences across groups were compared with one-way ANOVA or Chi-square tests for normally distributed continuous and categorical variables, respectively. Tests for linear trend of nutrient intakes across DDS categories were performed by assigning the midpoint values of DDS categories and treating the variable as continuous in a separate regression model, prior to that, values of nutrient intakes were transformed to log to reach normality. Multinomial logistic regression models were conducted to investigate the association of DDS scores with self-reported memory status (OK, good, or bad; participants whose memory was OK as the comparison group) and memory change in the past 12 months (stayed the same, improved, or deteriorated; participants whose memory stayed the same as the comparison group). Linear regression models were carried out to explore the association between DDS and memory scores. Multivariate models were conducted, and factors with $P < 0.05$ in the univariate analyses were included. In the first model, covariate including age, living region, education level, and income, were adjusted. In the second model, smoking status, alcohol consumption, and history of diabetes were additionally adjusted. To confirm the robustness of our findings, sensitivity analyses were conducted by (1) additional adjustment of gender, history of infarction, and history of hypertension; (2) excluding participants whose income information were missing at baseline. We also did subgroup analyses according to gender (men or women) and age (<65 or $\geq$65 years). Statistics were conducted in R 4.0.2. The multinomial logistic regressions were conducted with R package nnet (Venables and Ripley, 2002). All *P*-values were two-sided, and statistical significance was defined as $P < 0.05$.

RESULTS

Memory Status

A total of 4356 participants were included in our analysis, with an average age of (61.9 $\pm$ 7.9) years. The percentages of participants who thought their memory was OK, bad, and good were 43.3, 24.3, and 32.4%, respectively. There were 1.4% of participants reported memory improvement in the past 12 months and 47.2% reported memory decline. The average memory score among the participants was 12.8 $\pm$ 6.1.

DDS and Its Distribution Among Different General Characteristics

Average DDS in participants was 4.09 $\pm$ 1.13. **Table 1** showed the characteristics of participants across DDS categories. Participants with higher DDS were more likely to be relatively younger, living in southern China, having higher education and income, drinking less, never smoking, and having histories of diabetes. Men and women had similar DDS. Proportions of hypertension or myocardial infarction were similar in participants among different DDS categories.

Energy and Nutrient Intakes

Table 2 shows that participants in higher DDS categories had higher intakes of energy, protein, fat, dietary fiber, cholesterol, and most micronutrients (e.g., vitamin A, vitamin C, calcium). However, a negative trend was observed between DDS and intakes of carbohydrate, sodium, and manganese.

Association of DDS With Memory Status

Compared with participants who thought their memory was OK, higher DDS was associated with self-reported good memory and inversely associated with self-reported bad memory (**Table 3**). Comparing with participants whose memory stayed the same in the past 12 months, higher DDS was inversely associated with self-reported memory decline (**Table 4**). In addition, higher DDS was associated with higher memory scores (**Table 5**). The adjustment of covariates did not change the trends between DDS and outcomes. No association between DDS and memory improvement was observed (**Table 4**).

Sensitivity Analyses

In the sensitivity analyses, the associations of DDS with memory status, memory change, and memory score did not change after additional adjustment of gender, history of hypertension, and history of myocardial infarction (**Supplementary Table 1**) or excluding participants whose income information were missing at baseline (**Supplementary Table 2**).

Subgroup Analyses

In the subgroup analyses by gender, the associations were consistently between men and women and did not change appreciably compared with the results of the combined population. In the subgroup analyses by age (<65, $\geq$65 years), higher DDS was associated with self-reported good memory in participants aged below 65 years but not in those aged 65 years and above (**Supplementary Table 3**).

DISCUSSION

To our knowledge, the present study is the first one providing prospective evidence about dietary diversity and memory status in the Chinese population. Our study found that higher DDS was associated with self-reported good memory and higher memory score and was inversely associated with self-reported bad memory and memory decline.

TABLE 1 | General characteristics of participants according to DDS categories.

Variables	DDS categories				*P*
	(0,2]	(2,4]	(4,6]	(6,8]	
Number of participants	157	2246	1762	191	
Age (years)	65.4 ± 8.5	62.0 ± 7.9	61.4 ± 7.8	62.2 ± 8.1	<0.001
Gender					
Men	45.9	46.5	48.0	40.8	0.281
Women	54.1	53.5	52.0	59.2	
Living region					
Southern China	48.4	65.1	61.5	71.2	<0.001
Northern China	51.6	34.9	38.5	28.8	
Education					
Primary school or lower	83.4	64.9	39.6	16.8	<0.001
Lower middle school	11.5	22.3	30.7	24.1	
Middle school or above	5.1	12.8	29.7	59.2	
Income					
Low	74.5	53.2	23.8	3.1	<0.001
Middle	21.7	33.5	36.1	24.1	
High	3.8	13.3	40.1	72.8	
Alcohol consumption					
Never drink	71.3	70.2	65.6	69.1	0.002
<3 times a week	15.9	13.0	15.9	19.9	
≥3 times a week	12.7	16.8	18.4	11.0	
Smoking status					
Never smoke	65.6	65.8	68.0	82.7	<0.001
Used to smoke	7.6	5.3	6.4	4.7	
Currently smoke	26.8	28.9	25.6	12.6	
Hypertension					
No	59.9	58.4	57.7	56.5	0.897
Yes	40.1	41.6	42.3	43.5	
Diabetes					
No	98.7	95.3	92.5	92.7	<0.001
Yes	1.3	4.7	7.5	7.3	
Myocardial infarction[a]					
No	98.7	99.0	98.9	99.5	0.882
Yes	1.3	1.0	1.1	0.5	

Values are Means ± SDs for continuous variables and percentages for categorical variables unless stated otherwise. Differences across groups were compared by ANOVA and Chi-square tests for continuous and categorical variables, respectively. DDS, dietary diversity score. [a]*One missing value.*

In this study, we observed higher DDS was associated with better memory status and inversely associated with subjective memory decline. One of the explanations for the association could be explained by the positive trend between DDS and intakes of antioxidants. In the present study, we found participants with higher DDS had higher intakes of antioxidants, such as vitamin C and vitamin E. It has been long recognized that oxidative damage might lead impairment to the brain in aged people (Head, 2009), and cohort studies showed that intakes of antioxidants were inversely associated with the risks of Alzheimer disease (Engelhart et al., 2002) and dementia (Devore et al., 2010). Another explanation is that fish is an important part of DDS, and fish provides rich high-quality n-3 long-chain polyunsaturated fatty acids (n-3 PUFA). A cohort study in Chinese found higher fish consumption was associated with a slower decline in memory in adults aged 65 years and above (Qin et al., 2014). N-3 PUFAs, such as docosahexaenoic acid (DHA), not only participate in neurogenesis, synaptogenesis, and myelination (Joffre et al., 2014) but also reduce inflammation and oxidative stress in the brain (Avramovic et al., 2012; Laye et al., 2018). A systemic review and meta-analysis showed that DHA supplementation had a beneficial effect on memory function in older adults (Yurko-Mauro et al., 2015). In addition, our study observed a negative trend between DDS and the intake of manganese. Excess manganese in the brain can be neurotoxic (Dobson et al., 2004). An observational study in the Chinese elderly found that whole blood manganese was correlated with plasma amyloid-β peptides and manganese might be involved in the progress of Alzheimer's disease (Tong et al., 2014). Last but not least, participants with higher DDS had higher intakes of protein and most of the micronutrients, which

TABLE 2 | Energy and nutrients intake according to DDS categories.

	DDS categories				*P* for trend
	(0, 2]	(2, 4]	(4, 6]	(6, 8]	
Energy (kcal)	1540.5 (1249.8, 2072.8)	1788.2 (1386.1, 2281.2)	1915.0 (1566.6, 2390.8)	1956.4 (1637.1, 2324.8)	<0.001
Protein (g)	50.5 (44.8, 57.6)	58.7 (50.7, 68.7)	68.3 (59.5, 80.0)	82.3 (72.2, 93.7)	<0.001
Fat (g)	41.6 (29.6, 65.1)	74.3 (56.5, 93.8)	82.1 (67.1, 98.1)	89.2 (74.4, 100.0)	<0.001
Carbohydrate (g)	358.3 (304.2, 389.0)	281.5 (234.3, 324.4)	253.9 (214.0, 289.3)	236.3 (201.5, 265.1)	<0.001
Dietary fiber (g)	13.6 (10.0, 17.3)	12.2 (8.9, 16.4)	12.7 (9.5, 16.9)	14.3 (11.4, 19.2)	<0.001
Cholesterol (mg)	0.1 (0.0, 5.6)	163.2 (76.7, 299.7)	329.4 (203.2, 486.7)	512.7 (360.8, 651.5)	<0.001
Vitamin A (ugRE)	207.7 (83.9, 402.7)	334.4 (189.4, 598.5)	436.9 (286.7, 708.3)	639.2 (413.2, 893.6)	<0.001
Thiamin (mg)	0.8 (0.6, 1.0)	0.8 (0.7, 1.0)	0.8 (0.7, 1.0)	0.9 (0.8, 1.2)	<0.001
Riboflavin (mg)	0.5 (0.4, 0.6)	0.6 (0.5, 0.8)	0.8 (0.7, 1.0)	1.1 (1.0, 1.3)	<0.001
Niacin (mg)	9.9 (6.4, 12.5)	13.0 (10.0, 17.3)	14.3 (11.4, 17.8)	16.3 (13.7, 19.2)	<0.001
Vitamin C (mg)	69.0 (33.8, 100.8)	69.7 (41.2, 107.7)	78.0 (50.9, 114.9)	106.1 (77.5, 146.0)	<0.001
Vitamin E (mg)	27.3 (16.7, 41.1)	30.7 (20.5, 43.0)	31.6 (22.8, 42.3)	33.3 (24.9, 42.9)	<0.001
Calcium (mg)	293.9 (205.6, 380.5)	343.8 (262.0, 463.3)	434.6 (326.5, 582.7)	688.9 (540.1, 870.8)	<0.001
Phosphorus (mg)	886.7 (755.1, 989.2)	876.0 (766.4, 1023.0)	977.7 (850.6, 1136.6)	1196.8 (1078.4, 1339.7)	<0.001
Potassium (mg)	1568.5 (1280.9, 1805.0)	1595.5 (1297.2, 1966.9)	1801.9 (1514.4, 2171.9)	2237.7 (1971.6, 2572.7)	<0.001
Sodium (mg)	5016.2 (3399.3, 7131.3)	4817.8 (3421.4, 6838.7)	4343.6 (3216.0, 5959.5)	4433.9 (3297.4, 5580.9)	<0.001
Magnesium (mg)	321.5 (263.5, 366.8)	282.1 (236.0, 337.7)	294.2 (246.9, 348.5)	331.4 (300.0, 387.3)	<0.001
Iron (mg)	18.0 (14.9, 20.8)	18.0 (15.3, 22.1)	19.5 (16.4, 23.7)	20.8 (18.1, 25.0)	<0.001
Zinc (mg)	8.0 (6.6, 9.8)	9.6 (8.1, 11.2)	10.4 (9.0, 12.0)	11.7 (10.1, 13.3)	<0.001
Selenium (μg)	38.3 (20.5, 54.5)	36.2 (27.7, 47.3)	45.7 (36.5, 59.2)	61.0 (48.8, 77.2)	<0.001
Copper (mg)	1.3 (1.1, 1.7)	1.5 (1.2, 1.8)	1.6 (1.3, 2.0)	1.9 (1.5, 2.4)	<0.001
Manganese (mg)	5.9 (4.8, 7.0)	5.3 (4.2, 6.3)	4.9 (4.0, 5.9)	4.7 (3.8, 5.8)	<0.001

Values are medians and quartiles unless stated otherwise. Values of nutrient intake were expressed values per 2000 kcal, except for energy. DDS, dietary diversity score. Tests for linear trend across categories were performed by assigning the midpoint values of DDS categories and treating the variable as continuous in a linear regression model, prior to that, values of nutrient intakes were transformed to log to reach normality.

TABLE 3 | Association between DDS and self-reported memory status.

	DDS categories				Continuous
	(0, 2]	(2, 4]	(4, 6]	(6, 8]	
Good					
Crude	Ref	3.06 (1.77, 5.29)***	4.30 (2.48, 7.44)***	5.09 (2.75, 9.44)***	1.26 (1.18, 1.34)***
Model 1	Ref	2.58 (1.48, 4.49)**	3.02 (1.72, 5.29)***	3.13 (1.65, 5.94)***	1.14 (1.07, 1.23)***
Model 2	Ref	2.57 (1.48, 4.48)**	3.02 (1.73, 5.30)***	3.24 (1.71, 6.15)***	1.15 (1.07, 1.24)***
Bad					
Crude	Ref	0.78 (0.55, 1.10)	0.51 (0.36, 0.73)***	0.27 (0.15, 0.49)***	0.76 (0.70, 0.81)***
Model 1	Ref	0.96 (0.67, 1.38)	0.72 (0.49, 1.05)	0.40 (0.21, 0.76)**	0.82 (0.75, 0.89)***
Model 2	Ref	0.95 (0.66, 1.37)	0.71 (0.48, 1.04)	0.39 (0.20, 0.74)**	0.82 (0.75, 0.89)***

*Values are odds ratios (95%CIs). **P < 0.01; ***P < 0.001. DDS, dietary diversity score; Ref, reference. Nominal logistic regression models were conducted to investigate the association between DDS and self-reported memory status (OK, good, or bad), taking the participants whose memory was OK as the comparison group. Multivariate models were adjusted for: Model 1: age (continuous), living region (southern/northern China), education level (primary school or lower, lower middle school, or middle school or above), and income (low, middle, or high); Model 2: additionally included alcohol consumption (never drink, <3, or ≥3 times a week), smoking status (never smoke, used to smoke, currently smoke), and history of diabetes (yes/no).*

could promote the overall health of participants and slow down memory loss.

One interesting finding in this study is, in the subgroup analysis, we observed higher DDS was positively associated with self-reported good memory in participants aged below 65 years, however, in those aged 65 years and above, the association was insignificant. We assumed that in elderly people, degenerative diseases were the main cause of memory loss. Elderly people were vulnerable to age-related, degenerative diseases, which might influence memory directly [e.g., diabetes (Gregg et al., 2000), stroke (Kuzma et al., 2018)] or indirectly [e.g., hospitalization (Wilson et al., 2012)]. However, it worth noting that, the current study also showed a relatively lower DDS in elders aged 65 years and above

TABLE 4 | Association between DDS and self-reported memory change in the past 12 months.

	DDS categories				Continuous
	(0, 2]	(2, 4]	(4, 6]	(6, 8]	
Improved					
Crude	Ref	1.38 (0.19, 10.30)	1.57 (0.21, 11.66)	2.06 (0.24, 17.93)	1.08 (0.87, 1.35)
Model 1	Ref	1.46 (0.19, 11.11)	1.53 (0.20, 11.94)	1.75 (0.18, 16.55)	1.02 (0.79, 1.32)
Model 2	Ref	1.45 (0.19, 11.02)	1.43 (0.18, 11.25)	1.56 (0.16, 14.82)	1.00 (0.77, 1.29)
Deteriorated					
Crude	Ref	0.58 (0.42, 0.82)**	0.42 (0.30, 0.59)***	0.22 (0.14, 0.34)***	0.77 (0.73, 0.81)***
Model 1	Ref	0.74 (0.52, 1.05)	0.66 (0.46, 0.94)*	0.38 (0.23, 0.62)***	0.86 (0.81, 0.92)***
Model 2	Ref	0.73 (0.51, 1.03)	0.64 (0.45, 0.92)*	0.35 (0.22, 0.58)***	0.85 (0.80, 0.91)***

*Values are odds ratios (95%CIs). *P < 0.05;**P < 0.01;***P < 0.001. DDS, dietary diversity score; Ref, reference. Nominal logistic regression models were conducted to investigate the association between DDS and self-reported memory change in the past 12 months (stayed the same, improved, or deteriorated), taking the participants whose memory stayed the same in the past 12 months as the comparison group. Multivariate models were adjusted for: Model 1: age (continuous), living region (southern/northern China), education level (primary school or lower, lower middle school, or middle school or above), and income (low, middle, or high); Model 2: additionally included alcohol consumption (never drink, <3, or ≥3 times a week), smoking status (never smoke, used to smoke, currently smoke), and history of diabetes (yes/no).*

TABLE 5 | Association between DDS and memory score.

	DDS categories				Continuous
	(0, 2]	(2, 4]	(4, 6]	(6, 8]	
Crude	Ref	0.74 (− 0.23, 1.71)	2.67 (1.69, 3.64)***	5.66 (4.39, 6.93)***	1.26 (1.10, 1.41)***
Model 1	Ref	−0.46 (− 1.39, 0.46)	0.54 (− 0.42, 1.49)	2.85 (1.59, 4.12)***	0.73 (0.55, 0.90)***
Model 2	Ref	−0.44 (− 1.36, 0.49)	0.58 (− 0.38, 1.53)	2.96 (1.69, 4.22)***	0.74 (0.56, 0.91)***

*Values are β (95%CIs). ***P < 0.001. DDS, dietary diversity score; Ref, reference. Memory scores was calculated based on a subset from the Telephone Interview for Cognitive Status-modified, including immediate and delayed free-recall test (10 words) and the Serial 7s test. 98.6% of participants took this test. Linear regression models were conducted to investigate the association between DDS and memory scores. Multivariate models were adjusted for: Model 1: age (continuous), living region (southern/northern China), education level (primary school or lower, lower middle school, or middle school or above), and income (low, middle, or high); Model 2: additionally included alcohol consumption (never drink, <3, or ≥3 times a week), smoking status (never smoke, used to smoke, currently smoke), and history of diabetes (yes/no).*

(4.00 ± 1.18) compared with that of participants younger than 65 years (4.13 ± 1.10, $P_{t-test} < 0.001$). Nutrition interventions are needed to help elders to achieve a more diverse diet which may be beneficial to cope with the age-related memory decline.

DDS is widely used as an index of nutrient adequacy. Our study found higher DDS was associated with higher intakes of most macronutrients and micronutrients, which was consistent with previous studies (Tavakoli et al., 2016; Cano-Ibanez et al., 2019). Individuals who enjoyed higher dietary diversity had a lower intake of sodium. High dietary sodium is considered as a risk factor of hypertension (Karppanen and Mervaala, 2006), and dietary sodium related hypertension is an important public health concern in China (Liu, 2009). The prevalence of hypertension in Chinese aged 60 years and above in 2012 was 58.9% (National Health and Family Planning Commission of the People's Republic of China, 2016), however, it is reported that 77.64% of Chinese adults aged 60 years and above consumed more salt than the recommendations of Chinese dietary guidelines in 2015 (Wang et al., 2016; Jiang et al., 2019). Improving dietary diversity might be regarded as one of the components of the strategy of hypertension prevention. Besides, we found among participants, higher DDS was associated with other healthy lifestyles, including drinking less and smoking less, which may provide comprehensive effects on memory health. Findings also have implications for nutrition education, suggesting the need of increasing overall health awareness.

The strength of this study included population-based sample and prospective design, which lend strength to interferences. There are several limitations. First, although medical histories (hypertension, diabetes, and myocardial infarction) were considered in analysis, other comorbidities and life events might also have negative impacts on participants' memory. Second, as the dietary data were self-reported, participants might overestimate the intake of some foods and underestimate some other foods because of social desirability (Hebert, 2016). Besides, the gender difference was observed in these biases (Hebert et al., 1997). In the present study, we did subgroup analysis by gender, and the trends of DDS and memory in men and women was consistent with the whole population. Third, some participants chose "unknown" when reporting their memory status. As the survey did not further inquiry about the reason for their choices, we excluded these individuals from analysis. Fourth,

assessment of subjective memory status was based on self-report not standard scales. Further studies with a validated scale to measure the memory status are welcome.

CONCLUSION

Our study revealed that higher DDS was associated with better memory status and was inversely associated with self-reported memory decline in Chinese adults. Based on the findings of the present study, we proposed the recommendation of increasing diversity of diet in elderly people to promote memory health and delay memory decline progression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Boards at the University of North Carolina at Chapel Hill and the National Institute of Nutrition and Food Safety, China Centre for Disease Control and Prevention. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ, AZ, and YZ: conceptualization. JZ, WW, and CY: methodology. JZ and AZ: writing original draft. ZR, MW, PW, and YZ: review and editing. All the authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.580760/full#supplementary-material

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GLT-1 Knockdown Inhibits Ceftriaxone-Mediated Improvements on Cognitive Deficits, and GLT-1 and xCT Expression and Activity in APP/PS1 AD Mice

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Objective: Glutamate transporter-1 (GLT-1) and system x_C^- mediate glutamate uptake and release, respectively. Ceftriaxone has been reported to upregulate GLT-1 expression and improve cognitive decline in APP/PS1 mice. The aim of the present study was to elucidate the role of GLT-1 in ceftriaxone-mediated improvement on cognitive deficits and associated changes in xCT (catalytic subunit of system x_C^-) expression and activity using GLT-1 knockdown APP/PS1 mice.

Methods: GLT-1 knockdown (GLT-1$^{\pm}$) mice were generated in C57BL/6J mice using the CRISPR/Cas9 technique and crossed to APP/PS1 mice to generate GLT-1$^{\pm}$APP/PS1 mice. The cognition was evaluated by novel object recognition and Morris water maze tests. GLT-1 and xCT expression, GLT-1 uptake for glutamate, and glutathione levels of hippocampus were assayed using Western blot and immunohistochemistry, ^{3}H-glutamate, and glutathione assay kit, respectively.

Results: In comparison with wild-type mice, APP/PS1 mice exhibited significant cognitive deficits, represented with poor performance in novel object recognition and Morris water maze tests, downregulated GLT-1 expression and glutamate uptake. Ceftriaxone treatment significantly improved the above impairments in APP/PS1 mice, but had negligible impact in GLT-1$^{\pm}$APP/PS1 mice. The xCT expression increased in APP/PS1 and GLT-1$^{\pm}$APP/PS1 mice. This upregulation might be a compensatory change against the accumulated glutamate resulting from GLT-1 impairment. Ceftriaxone treatment restored xCT expression in APP/PS1 mice, but not in GLT-1$^{\pm}$APP/PS1 mice. Glutathione levels decreased in APP/PS1 mice in comparison to the wild-type group. After ceftriaxone administration, the decline in glutathione level was restored in APP/PS1 mice, but not in GLT-1$^{\pm}$APP/PS1 mice.

Conclusion: Ceftriaxone improves cognitive impairment of APP/PS1 mice by upregulating GLT-1-mediated uptake of glutamate and co-regulation of GLT-1 and xCT in APP/PS1 mice.

Keywords: ceftriaxone, GLT-1, GLT-1 knockdown, glutamate uptake, xCT, glutathione level, APP/PS1 mice

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INTRODUCTION

Alzheimer's disease (AD) is a common age-related neurodegenerative disease with learning and memory impairment, and progressive dementia (Selkoe, 2002). Although the exact pathogenesis of AD has not been fully elucidated, numerous studies have shown that dysfunction of the glutamatergic neuronal system plays an important role in the pathogenesis of AD (Hardy et al., 1987; Masliah et al., 2000; Hynd et al., 2004; Scott et al., 2011). Glutamate, the primary neurotransmitter of glutamatergic neuronal system, exerts an essential function in learning and memory (Willard and Koochekpour, 2013). Either dysfunction in glutamate reutilization or overaccumulation of glutamate in extracellular space in the brain, resulting in excitotoxicity and leading to synapse loss, would impair synaptic transmission and connection, and thus induce cognitive decline (Francis, 2003; Hynd et al., 2004; Talantova et al., 2013; Audrain et al., 2016). The concentration of glutamate in the synaptic cleft is principally regulated by glutamate transporters, especially glutamate transporter-1 (GLT-1), which accounts for a major role in glutamate uptake and exerts an important role in reutilization of glutamate as a neurotransmitter and prevention of glutamate excitotoxicity (Haugeto et al., 1996; Rothstein et al., 1996). Impairment of GLT-1 expression and/or uptake and the subsequent dysregulation in glutamate level exerts a crucial role in the pathogenesis of AD. For example, amyloid-peptide (Aβ) reduces glutamate uptake by decreasing GLT-1 expression or inducing mislocalization and endocytosis of GLT-1 in astrocytes of AD model animals (Scimemi et al., 2013; Tong et al., 2017). Moreover, GLT-1 expression in the hippocampus of 3xTg-AD model mouse was found to be significantly downregulated (Zumkehr et al., 2015). Glutamate uptake activity in astrocytes derived from the cortex of patients with AD is also significantly reduced (Liang et al., 2002; Scott et al., 2011). Transgenic AD mice with GLT-1 knockdown show aggravated cognitive impairment (Mookherjee et al., 2011). Moreover, dihydrokainic acid, a GLT-1 inhibitor, impairs memory performance in rat and mouse (Bechtholt-Gompf et al., 2010; Tian et al., 2019). The reports suggest that regulation of GLT-1 expression and uptake activity may be protective against cognitive deficits in AD.

Ceftriaxone (Cef), a β-lactam antibiotic, has been reported to increase GLT-1 expression significantly, and this increase can provide neuroprotection in animal models of amyotrophic lateral sclerosis (Rothstein et al., 2005), Parkinson's disease (Hsu et al., 2015; Hsieh et al., 2017), brain ischemic preconditioning (Chu et al., 2007; Harvey et al., 2011; Hu et al., 2015; Krzyzanowska et al., 2017), pain (Hu et al., 2010), and seizure (Soni et al., 2015; Hussein et al., 2016). Recently, we have reported that Cef can improve learning and memory impairment in early stage APP/PS1 AD mouse, accompanied by an upregulation of GLT-1 expression (Fan et al., 2018). The finding suggests a possibility that GLT-1 upregulation mediated the above effect after Cef treatment. Here, to prove the possibility, we used partial GLT-1 knockout APP/PS1 (GLT-1$^{\pm}$APP/PS1) AD mouse to investigate the influence of Cef on learning and memory deficits by novel object recognition and Morris water maze tests, GLT-1 expression, and glutamate uptake capacity of the AD mice.

System x_c^- is a cystine/glutamate antiporter, which imports cystine in exchange for intracellular glutamate (Bannai and Kitamura, 1980; Lo et al., 2008; Lewerenz et al., 2013). The exchange promotes the synthesis of glutathione and increases antioxidant activity in astrocytes while simultaneously participating in the maintenance of glutamate homeostasis. The activity of the cystine/glutamate antiporter is stimulated by an increased activity of glutamate transporter (Rimaniol et al., 2001; Lewerenz et al., 2006). So, the present study also aimed to observe changes in xCT expression (the catalytic subunit of system x_c^-) and levels of glutathione, to reflect the activity of System x_c^- in GLT-1$^{\pm}$APP/PS1 AD mouse, and the influence of Cef on its expression and activity.

MATERIALS AND METHODS

Experimental Animals, Grouping, and Protocols

Ninety-seven male mice aged 6 months old (25–30 g in weight) were used. The mice were bred in the animal facilities of the Laboratory Animal Center of Hebei Medical University, China. The animals were housed in a temperature-controlled facility, at 23°C, with a 12-h light/dark cycle and free access to food and water. All water, food, and padding used in the cages were sterilized by autoclaving. All experimental procedures were performed according to the "ARRIVE" guidelines (McGrath and Lilley, 2015) and approved by the Animal Care and Use Committee of the Hebei Medical University, China. All efforts were done to minimize suffering and numbers of mice.

The animals used in the present work include wild-type (C57BL/6J), APP/PS1, and GLT-1$^{\pm}$APP/PS1 mice. The APP/PS1 mice were purchased from the Chinese Academy of Medical Sciences. This mouse overexpresses human amyloid precursor protein harboring the Swedish (K594M/N595L) mutation and presenilin 1 deleted in exon 9 in a C57BL/6J genetic background, and shows pathological phenotypes of AD, including learning and memory impairment at 3–5 months of age and several senile plaques at 12 months of age (Webster et al., 2014). The GLT-1$^{\pm}$APP/PS1 mouse was obtained by crossing the APP/PS1 and GLT-1$^{\pm}$ mice, as described previously (Mookherjee et al., 2011). Briefly, the GLT-1$^{\pm}$ mouse was first generated in a C57BL/6J mouse using the CRISPR/Cas9 technique. The NGG in the PAM locus of *slcla2* (reference sequence: NC_000068.7) was selected for designing sgRNA. sgRNA1 (GCAAGGGAATGACTCCTGGGAA) and sgRNA2 (ACAGGTGCCTCAATGGCA) were selected from the upstream and downstream regions of the second exon, respectively. A 480-bp sequence, containing the shared second exon, was knocked-out, which resulted in transcoding mutations and premature termination (**Figure 1**). After screening and sequencing, the GLT-1$^{\pm}$ mouse, with reduced GLT-1 expression levels, was obtained. The GLT-1$^{\pm}$ mouse has a normal life span compared to the wild-type mouse (Tanaka, 1997). Thereafter, the GLT-1$^{\pm}$ mouse was

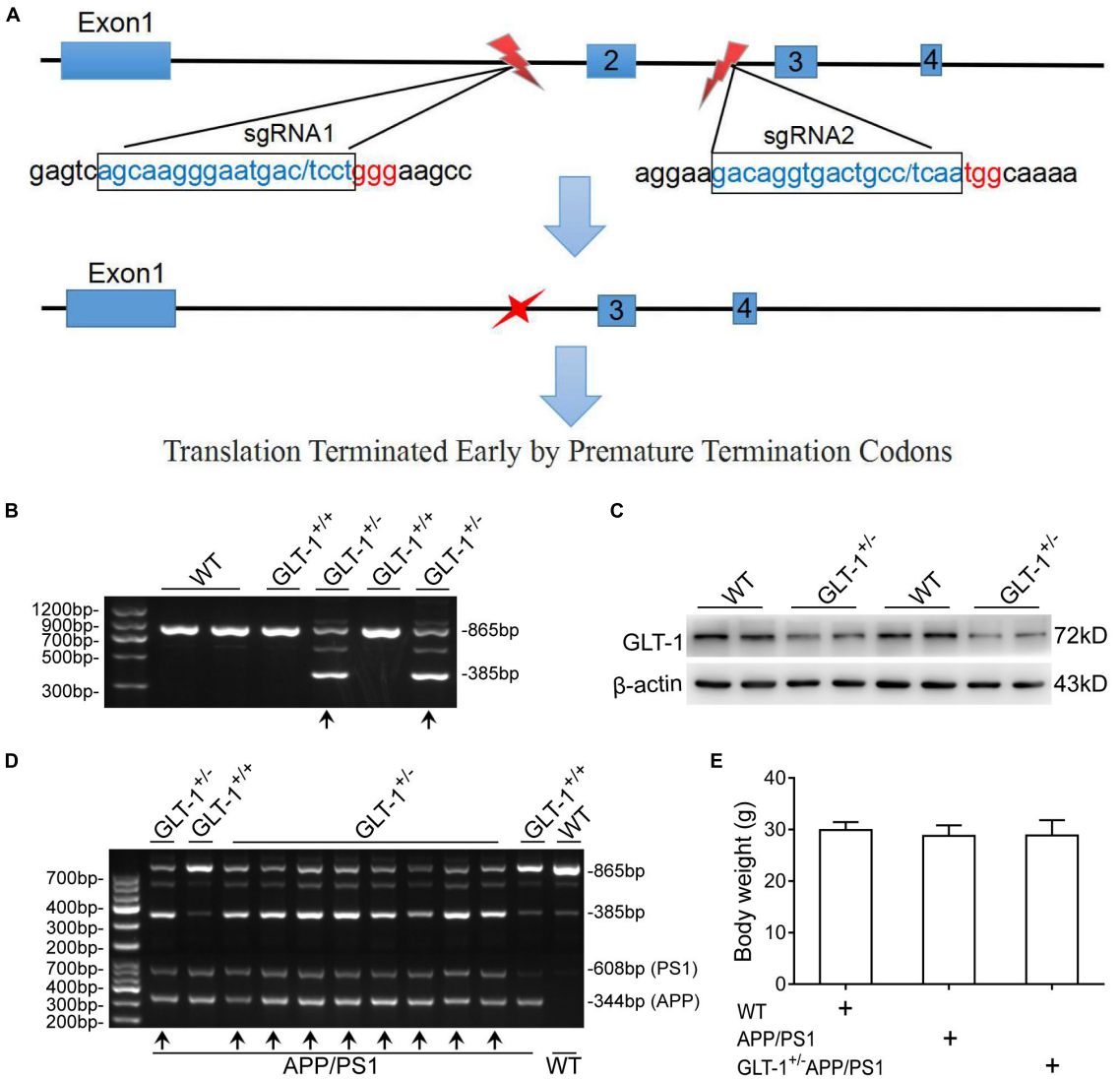

FIGURE 1 | The generation and identification of glutamate transporter-1 (GLT-1)$^{\pm}$APP/PS1 mice. **(A)** Schematic of the genetic modification technique used. SgRNA1 and sgRNA2 were designed as shown. One incision was made on each side of the second exon to knock out a 480 -bp sequence, which includes the second exon. This modification induces transcoding mutations of GLT-1 that terminate prematurely. **(B)** PCR-based genotyping of mice offspring using tail tissue showing genotype of GLT-1 knockdown (the bands have been indicated by upward arrows). **(C)** Western blot analysis showing downregulated GLT-1 expression in the hippocampus of GLT-1$^{\pm}$ mice compared with wild-type mice. **(D)** PCR-based genotype identification of the GLT-1$^{\pm}$APP/PS1 mice offspring, clearly showing GLT-1 knockdown and APP/PS1 transgenic features (bands have been indicated by upward arrows). **(E)** There is no difference in the body weight of wild-type, APP/PS1, and GLT-1$^{\pm}$APP/PS1 mice at 6 months old.

crossed with the transgenic APP/PS1 mouse to generate the GLT-1$^{\pm}$APP/PS1 mouse. The gene-modified pups were identified by PCR using DNA extracted from the tail tissue of the mouse at 3 weeks of age. Only mice that showed clear APPswe/PS1dE9 transgenic features and GLT-1$^{\pm}$ knockdown features (**Figure 1**) were used in the study.

The study designed included wild-type (C57BL/6J), APP/PS1, GLT-1$^{\pm}$APP/PS1, Cef + APP/PS1, and Cef + GLT-1$^{\pm}$APP/PS1 groups. The mice in Cef + APP/PS1 and Cef + GLT-1$^{\pm}$APP/PS1 groups were administrated Cef by peritoneal injection for 14 days, once a day at a dose of 200 mg/kg, which was determined according to previous reports (Rothstein et al., 2005; Hu et al., 2015; Fan et al., 2018). The mice in other groups were administrated normal saline as solvent control, using the same protocols. After completion of the administration, all mice went through behavioral tests. Novel object recognition and Morris water maze tests are common methods in evaluating cognitive deficits of AD animals in non-spatial working memory ability and spatial working memory task (Morris et al., 1982; Vorhees and Williams, 2014; Balderas et al., 2015). Thus, the mice in the present study received novel object recognition and Morris water maze tests separately. After completing the behavioral test, the mice were euthanized, and the hippocampi were collected under anesthesia for the assays of GLT-1 and xCT expression, GLT-1

uptake, and GSH level. Details of the animal number for each test are shown in **Table 1**.

PCR for Identification of Gene Modification

The specific primers used were as follows:

APP: 5′-GACTGACCACTCGACCAGGTTCTG-3′
5′-CTTGTAAGTTGGATTCTCATATCCG-3′
PS1: 5′-AATAGAACGGCAGGAGCA-3′
5′-GCCA-TGAGGGCACTAATCAT-3′
GLT-1: 5′GAGGGAAAGTTTGAGTTACCAGC-3′
5′-CATGTTCACCCTCACAGCAACT-3′

PCR was conducted under the following conditions:

For APP, 94°C for 3 min, 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles, followed by extension at 72°C for 10 min.
For PS1, 94°C for 3 min, 94°C for 40 s, 52°C for 40 s, and 72°C for 1 min for 30 cycles, followed by extension at 72°C for 10 min.
For GLT-1, 94°C for 5 min, 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 30 cycles, followed by extension at 72°C for 7 min.
The lengths of PCR products were as follows: APP, 344 bp; PS1, 608 bp; GLT-1^{+}, 865 bp; GLT-1^{-}, 385 bp. Samples were loaded in 2% agarose gels. A gel imaging and analysis system (GENE, United Kingdom) was used for imaging.

Novel Object Recognition Test

The test was run in four black training boxes (40 cm × 40 cm × 30 cm), which were placed in a sound proof lucifuge cabinet. The whole experiment was recorded by a video camera above the chamber. The data were collected and analyzed using the DigBehv Animal Behavior Analysis Software (Shanghai Jiliang Software Technology Co., Ltd., China). The test lasted for 5 days and was divided into habituated stage, acquisition stage, and retention stage, as reported previously (Blurton-Jones et al., 2009). The habituated stage lasted for 3 days; mice were placed in a box, without any objects, for 5 min/day, so they could adapt to the environment. In the acquisition stage (on day 4), mice were exposed to two wooden cubes (3 cm × 3 cm × 3 cm) for 3 min. The cubes were fixed to the floor of the box, 8 cm from the wall, and object location was counterbalanced during the behavior test. The mice were placed in the training box with their noses equidistant from the two objects. In the retention stage (on day 5), one of the cubes was randomly replaced with a hemisphere (4 cm in diameter), which was made of the same material and was as attractive as the cube. The mice were allowed to explore the two objects freely for 3 min. The exploratory behavior of the mice was as follows: the tip of the nose of the mice touched the object or was within 2 cm of the object. The recognition ability of the mice to new objects was evaluated using the recognition index, which was calculated as: time spent on novel object/(time spent on novel object + time spent on familiar object) × 100%. After each trial, the floor of the box and the objects were wiped with 70% ethanol to dispel any preference due to smell.

TABLE 1 | The details of the animal number in each test.

Group	Number of animals in each test						
	NOR	MWM	GLT-1 exp		GLT-1 uptake	xCT (WB)	GSH level
			WB	IHC			
Wild type	8	10	6	6	6	6	6
APP/PS1	9	13	6	6	6	6	6
GLT-$1^{\pm}$APP/PS1	8	10	6	6	6	6	6
Cef + APP/PS1	8	12	6	6	6	6	6
Cef + GLT-$1^{\pm}$APP/PS1	6	10	6	6	6	6	6

NOR, novel objective recognition test; MWM, Morris water maze test; exp, expression; WB, western blot; IHC, immunohistochemistry; GSH, glutathione; GLT-1, glial glutamate transporter-1; xCT, catalytic subunit of system x_c^-; Cef, Ceftriaxone. WB for GLT-1 and xCT expression and the assay of GSH level were performed using the same six animals, from which, the right hippocampus was used for WB of GLT-1 and xCT expression, and the left hippocampus was used for the assay GSH level. The animals subjected to the assays of GLT-1 expression and uptake, xCT expression and GSH level received behavioral tests of which three animals received the NOR test, and three received the MWM test, except three animals in the assay of GLT-1 uptake in Cef + GLT-$1^{\pm}$APP/PS1 group did not receive the NOR test, because of trouble of the equipment.

Morris Water Maze Test

The test was conducted in a sound proof room in which several visual cues were set in fixed positions. The plastic tank was 120 cm in diameter and 50 cm high, filled with water, in which the temperature was maintained at 20 ± 1°C during the experiment. An escape platform with a diameter of 9 cm was placed in the first quadrant, 1.0 cm below the water surface. A video camera and DigBehv Animal Behavior Analysis Software (Shanghai Jiliang Software Technology Co., Ltd., China) were used for tracking the search bias of mice and analyzed their behavioral performance. Space navigation and retention tests were performed. The navigation test lasted for 4 days with five trials per day. At every trial, mice were put in water, using one of the four start points randomly, with their nose toward the wall and allowed to swim and search the platform. If the mice failed to find the platform successfully in 60 s, the mice were guided to the platform and allowed to remain there for 15 s. The time that the mouse took to climb onto the platform within 60 s and stay there for more than 3 s was determined as escape latency. The average escape latency of five trials was used for statistical analysis. The retention tests were conducted on the fifth navigation of the fourth day. After removing the platform, mice were put in the water from the contralateral quadrant of the platform and allowed to swim for 60 s. The time spent in the target quadrant and the times crossing the platform site were determined as reflection of the retention performance.

Western Blot Analysis for GLT-1 and xCT Expression

The mice were decapitated under anesthesia. The hippocampus was dissected out and homogenized in 10 volumes of lysis buffer, contained protease inhibitors (Roche, Germany). The

homogenates were centrifuged at 12,000 rpm/min for 15 min at 4°C and supernatants were analyzed. The concentration of protein of the supernatants was determined using BCA assay kit (Solarbio, Beijing, China). Thereafter, samples containing protein of 30 μg were loaded on 12% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States) at 21 V for 1 h. Thereafter, the membranes were blocked in 5% bovine serum albumin (BioFroxx, Germany) at 37°C for 1 h, and incubated at 4°C for 12 h with primary antibodies against GLT-1 (polyclonal antibody derived from guinea pig, 1:2,000, Cat. No.: AB1783, Lot No.: 2987435, Millipore, United States), xCT (monoclonal antibody derived from rabbit, 1:2,000, Cat. No.: ab175186, Lot: GR3235736-1, Abcam, United States), and β-actin (monoclonal antibody derived from rabbit, 1:10,000, Cat. No.: AC026, Lot: 9100026001, ABclonal, United Kingdom). Subsequently, the membranes were washed with TBST and incubated at 37°C for 1 h with secondary antibodies for GLT-1 (anti-guinea pig IgG labeled with biotin derived from goat, 1:3,000, Cat. No.: 16-17-06, Lot No.: 130670, KPL, United States), and xCT and β-actin (HRP-labeled anti-rabbit IgG derived from goat, 1:2,000, Cat. No.: 074-1506, Lot No.: 140740, KPL, United States). For GLT-1, an additional incubation with streptavidin conjugated with HRP (1:2,000, Cat. No.: 43-4323, Lot No.: 1513798A, Invitrogen, United States) was conducted at 37°C for 1 h. All membranes were visualized using an ECL reagent (Vazyme, Nanjing, China) after TBST washes. The integral optical density (IOD) of the band was measured with an analysis tool (Alpha Imager, United States). The ratio of IOD of the target protein band to the β-actin band was calculated as the relative change in expression of target protein.

Immunohistochemistry for GLT-1 Expression

The mice were anesthetized with isoflurane, and perfused via the ascending aorta with normal saline and 4% paraformaldehyde. The brain was removed, and a coronal slice of 3 -mm thickness, including the bilateral hippocampus, was cut. After fixation with 4% paraformaldehyde overnight, the brain slices were embedded in paraffin. Sections of 4-μm thickness were cut. After deparaffinization with xylene and hydration in a descending series of alcohol, sections were incubated with hydrogen peroxide (3%, Lot: AH07183973, Bioss, Beijing, China) for 20 min to remove endogenous peroxidase, heated in a microware in citrate buffer (0.01 M, pH = 6.0) for 18 min for antigen repairing, and then blocked in 10% goat serum (Lot: AH07183973, Bioss, Beijing, China) for 40 min at 37°C. Thereafter, the sections were incubated with primary antibodies against GLT-1 (the same as that used for Western blot, 1:500) overnight at 4°C. After PBS washes, the sections were incubated with secondary antibody (anti-guinea pig IgG labeled with biotin derived from goat, Lot No.: AH07183973, Bioss, Beijing, China) for 1 h at 37°C. Then sections were washed with PBS and incubated with streptavidin working solution conjugated with horseradish peroxidase (Lot: AH07183973, Bioss, China) for 1 h at 37°C. Detections were done using DAB kit (Lot: K166622C, Zhongshan, China). The integral optical density (IOD) of immunostaining was analyzed by Image J to determine the relative expression of GLT-1.

Glutamate Uptake

The GLT-1 uptake capacity was assayed with ^{3}H-glutamate, as described previously (Hu et al., 2015). Briefly, the hippocampus of the mouse was rapidly separated and cut into a small piece in trypsin (0.25%) at 37°C. After the digestion by trypsin for 10 min, Hank's solution containing calcium and magnesium was added to the samples to block the digestion. The samples were blown 40–60 times in Hank's solution and then centrifuged at 1,000 rpm/min for 10 min at 4°C. After discarding the supernatant, the cell pellets were resuspended in 500 μl of Hank's solution for GLT-1 uptake assay. The samples containing 50 μg of protein, determined by the BCA protein assay kit, were incubated with ^{3}H-glutamate solution (100 μl, 50 nmol/L) at 37°C for 15 min, in the absence (total glutamate uptake) or presence (non-specific glutamate uptake) of dihydrokainate (0.18 mmol/L, Lot: 064M4608V, Sigma, United States), the specific inhibitor of GLT-1. An ice bath was used to terminate the reaction. The cell suspensions were centrifuged at 1,000 rpm/min for 10 min, and the supernatant was discarded. Thereafter, 1 ml of Hank's solution was added to the tube, and the cell suspensions were centrifuged again at 1,000 rpm/min for 10 min, with the supernatant being discarded. The cell pellets were lysed using NaOH (100 μl, 3 M) for 20 min. Thereafter, the lysed cell solution was transferred to Whatman filter. After drying at 60°C for 30 min, the filters were placed in a sample bottle (PerkinElmer, United States) containing 2 ml of scintillation liquid. Counts per minute (cpm) were determined by a liquid scintillation counter. The specific glutamate uptake capacity of GLT-1 was calculated through subtracting non-specific glutamate uptake from total uptake.

Glutathione Assay

The glutathione levels in the hippocampus were measured by fluorometry (Naletova et al., 2018; Piao et al., 2018) using an assay kit (Fluorometric, ab65322, Lot: GR3254829-6, Abcam, United States), according to protocols described in the instruction. The hippocampus of each animal was homogenized in 10 volumes of cell lysis buffer, on an ice bath, and then centrifuged at 12,000 rpm/min for 10 min at 4°C. The supernatant was collected and deproteinized as indicated in the protocol of the assay kit. The standard solution (100 μl) and sample (40 μl samples + 60 μl cell lysis buffer) were incubated with 2 μl of GST reagent and 2 μl of MCB for 5 min. Thereafter, the optical density of fluorescence was immediately measured at Ex/Em = 360/460 nm in the kinetic mode. The concentration of glutathione was expressed as μg/ml.

Statistics

SPSS 21 was used for statistical analysis. All the data were presented as mean ± standard deviation (SD). The escape latency in Morris water maze test was analyzed using repeated measures ANOVA. Other data were compared through one-way ANOVA combined with Tukey *post hoc* test. A value of $p < 0.05$ was considered statistically different.

RESULTS

GLT-1 Knockdown Inhibited the Improvement of Cef on Cognitive Deficit in APP/PS1 Mice

The novel object recognition tests indicated that [one-way ANOVA, $F(4,34) = 43.23$, $p < 0.05$], in comparison with wild-type mice, the cognitive index in APP/PS1 group significantly decreased ($p < 0.001$). Knockdown of GLT-1 in APP/PS1 mice (GLT-1$^{\pm}$APP/PS1 mice) further deteriorated the cognitive index compared with APP/PS1 mice ($p < 0.001$). Cef administration obviously increased the cognitive index in Cef + APP/PS1 mice compared with APP/PS1 mice ($p < 0.001$), whereas no changes were seen in Cef + GLT-1$^{\pm}$APP/PS1 mice compared with GLT-1$^{\pm}$APP/PS1 mice ($p = 0.660$) (**Figure 2A**).

Morris water maze tests revealed that [repeated measures ANOVA, $F(3,12) = 41.1$, $p < 0.05$], in comparison with wild-type mice, APP/PS1 mice exhibited longer escape latency in navigation trials ($p < 0.001$). GLT-1 knockdown further increased the escape latency of the APP/PS1 mice in the GLT-1$^{\pm}$APP/PS1 group in comparison with the APP/PS1 group ($p = 0.002$). During the retention test, APP/PS1 mice exhibited deteriorated retention performances represented with decreases in the time spent in the target quadrant (APP/PS1, $p = 0.001$) and times crossing the platform site (APP/PS1, $p = 0.002$) in comparison with the wild-type group. The GLT-1 knockdown in APP/PS1 mice has deteriorated more the time spent in the target quadrant ($p = 0.003$) and the times crossing the platform site ($p = 0.034$) than those in the APP/PS1 mice. Cef treatment significantly improved the performance in Morris water maze test, including escape latency ($p = 0.002$), the time spent in the target quadrant ($p < 0.001$), and the times crossing the platform site ($p = 0.001$) in the Cef + APP/PS1 mice compared with those in the APP/PS1 mice, while little change was seen in the Cef + GLT-1$^{\pm}$APP/PS1 mice compared with the GLT-1$^{\pm}$APP/PS1 mice (escape latency,

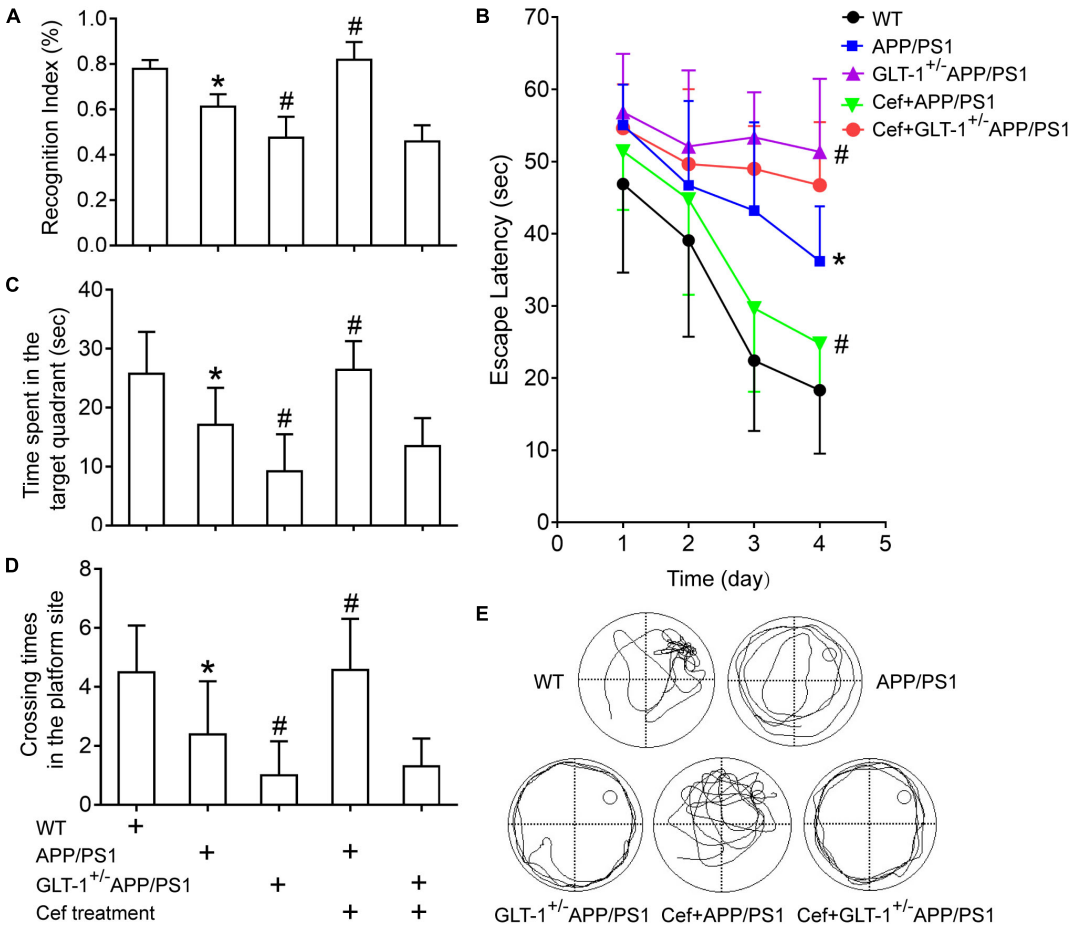

FIGURE 2 | The effect of GLT-1 knockdown on the cognitive improvement induced by Cef in 6 month-old APP/PS1 mice tested by novel object recognition **(A)** and Morris water maze tests **(B–E)**. *$p < 0.05$ vs. the wild-type group, #$p < 0.05$ vs. the APP/PS1 group. All the data were presented as mean ± standard deviation (SD) and the number of animals in each group in each test is shown in **Table 1**. Cef treatment increased the cognitive index in novel object recognition test **(A)**, decreased the escape latency **(B)**, and increased the time spent in the target quadrant and the times crossing the platform site **(C–E)** in Morris water maze test in the Cef + APP/PS1 group in comparison with the APP/PS1 group, while little effect in the Cef + GLT-1$^{\pm}$APP/PS1 group compared with the GLT-1$^{\pm}$APP/PS1 group was seen.

p = 0.171, time spent in the target quadrant p = 0.114, times of crossing the platform site p = 0.660) (**Figures 2B–E**).

Thus, novel object recognition and Morris water maze tests indicated that GLT-1 knockdown inhibited the improvement of Cef on cognitive impairment in APP/PS1 AD mice.

GLT-1 Knockdown Suppressed Cef-Induced Upregulation of GLT-1 Expression and Uptake Activity in APP/PS1 Mice

Immunohistochemical staining showed that [one-way ANOVA, $F(4,25) = 35.989$, $p < 0.05$], in wild-type mice, a large number of GLT-1 immunoreactive particles (brown) were distributed extensively in the entire hippocampus. APP/PS1 mice exhibited decreased GLT-1 immunostaining in the hippocampus, manifested with uneven distribution and flake deletions of the immunoparticles, and decreased IOD, in comparison with the wild-type group (p = 0.001). Knockdown of GLT-1 in the APP/PS1 mice (GLT-1 ± APP/PS1 group) further reduced GLT-1 expression compared with the APP/PS1 mice (p = 0.015). Cef treatment of the Cef + APP/PS1 group significantly increased the immunoreactivity of GLT-1 compared with the APP/PS1 group, as seen with more intense immunostaining and increased IOD of the immunoparticles in the hippocampus of the mice ($p < 0.001$ vs. APP/PS1 group). However, Cef treatment of the Cef + GLT-1$^{\pm}$APP/PS1 group did not increase the immunoreactivity of GLT-1 in comparison with the GLT-1$^{\pm}$APP/PS1 group (p = 0.929) (**Figures 3A–G**). Western blot assay [one-way ANOVA,

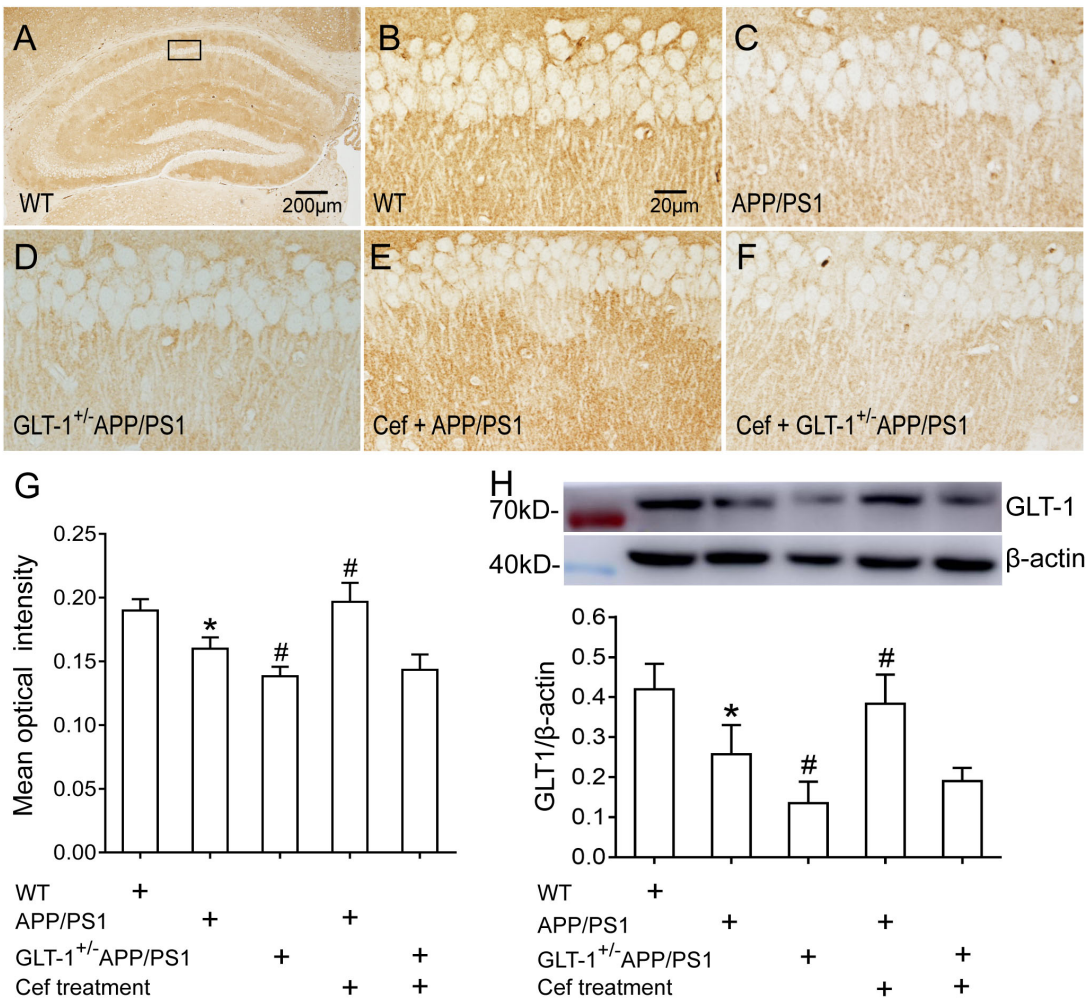

FIGURE 3 | The impact of GLT-1 knockdown on Cef-induced upregulation of GLT-1 expression in the hippocampus of 6-month-old APP/PS1 mouse evaluated by immunohistochemistry and Western blot. **(A–F)** Representative photomicrographs of immunohistochemical staining for GLT-1. The scale bar is 200 μm in **(A)**, and 20 μm in **(B)**, which is available for **(B–F)**. **(G)** A quantitative presentation of immunostaining by mean optical density. **(H)** Western blot analysis for GLT-1 expression, in which the top shows immunoreactive bands of GLT-1 and β-actin, the bottom bar graph is a quantitative presentation of the immunoreactive bands by integral optical density. *$p < 0.05$ vs. the wild-type group, #$p < 0.05$ vs. APP/PS1 group, n = 6 per group. All the data were presented as mean ± standard deviation (SD). Cef treatment upregulated GLT-1 expression in the Cef + APP/PS1 group compared with the APP/PS1 group, but had little effect in the Cef + GLT-1$^{\pm}$APP/PS1 group in comparison with the GLT-1$^{\pm}$APP/PS1 group.

$F(4,25) = 23.995$, $p < 0.05$] revealed similar changes as the immunohistochemistry assay on the impact of Cef on GLT-1 expression in APP/PS1 mice without or with knockdown of GLT-1 (**Figure 3H**).

The GLT-1 uptake assay showed that [one-way ANOVA, $F(4,25) = 34.931$, $p < 0.05$], in comparison with the wild-type group, the GLT-1 uptake capacity was significantly declined in the APP/PS1 group ($p = 0.031$), and the declines were more obvious in the GLT-$1^{\pm}$APP/PS1 group ($p = 0.017$ vs. APP/PS1 group). Cef treatment obviously reversed the decline in glutamate uptake by GLT-1 in the Cef + APP/PS1 group in comparison with the APP/PS1 group ($p < 0.001$), while there were no obvious changes in the Cef + GLT-$1^{\pm}$APP/PS1 group in comparison with the GLT-$1^{\pm}$APP/PS1 group ($p = 0.927$) (**Figure 4**).

The results revealed that GLT-1 knockdown inhibited Cef-induced upregulation of GLT-1 expression and uptake capacity in APP/PS1 mice.

The Impact of Cef on xCT Expression and Glutathione Level in APP/PS1 and GLT-$1^{\pm}$ APP/PS1 AD Mice

Western blot assay showed that [one-way ANOVA, $F(4,25) = 26.638$, $p < 0.05$] there was an obvious upregulation in xCT expression in APP/PS1 mice ($p < 0.001$) in comparison with the wild-type mice. GLT-1 knockdown in APP/PS1 mice (GLT-$1^{\pm}$APP/PS1 group) seemed to further increase xCT expression, although it was not statistically different in comparison to the APP/PS1 mice ($p = 0.523$). Cef treatment in APP/PS1 mice (Cef + APP/PS1 group) significantly restored xCT expression in comparison with the APP/PS1 group ($p = 0.013$). However, GLT-1 knockdown in APP/PS1 mice (GLT-$1^{\pm}$APP/PS1 group) prevented Cef from restoring xCT expression level ($p = 0.984$ vs. Cef + GLT-$1^{\pm}$APP/PS1 group) (**Figure 5A**).

Glutathione assay showed that [one-way ANOVA, $F(4,25) = 15.442$, $p < 0.05$] the glutathione level in the hippocampus of APP/PS1 mice and GLT-1 knockdown APP/PS1 mice (GLT-$1^{\pm}$APP/PS1 group) significantly declined in comparison to the wild-type group ($p = 0.005$ in APP/PS1 group and $p = 0.001$ in GLT-1 ± APP/PS1 group). Cef treatment in APP/PS1 mice (Cef + APP/PS1 group) increased glutathione level ($p < 0.001$ vs. APP/PS1 group). However, Cef could not increase glutathione levels in the Cef + GLT-$1^{\pm}$APP/PS1 group, in comparison with the GLT-$1^{\pm}$APP/PS1 group ($p = 0.995$) (**Figure 5B**).

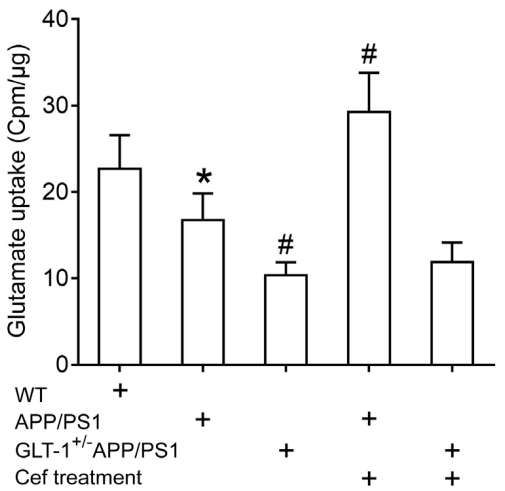

FIGURE 4 | The impact of Cef treatment on glutamate uptake of GLT-1 in the hippocampus and the impact of GLT-1 knockdown on the Cef-induced change in the uptake determined with ^{3}H-glutamate. *$p < 0.05$ vs. wild-type group, #$p < 0.05$ vs. APP/PS1 group, $n = 6$ per group. All the data were presented as mean ± standard deviation (SD). Glutamate uptake of GLT-1 reduced in APP/PS1 mice, and the knockdown of GLT-1 further exacerbated the decrease in the GLT-$1^{\pm}$APP/PS1 group. Cef treatment restored the decline in APP/PS1 mice, but not in GLT-$1^{\pm}$APP/PS1 mice.

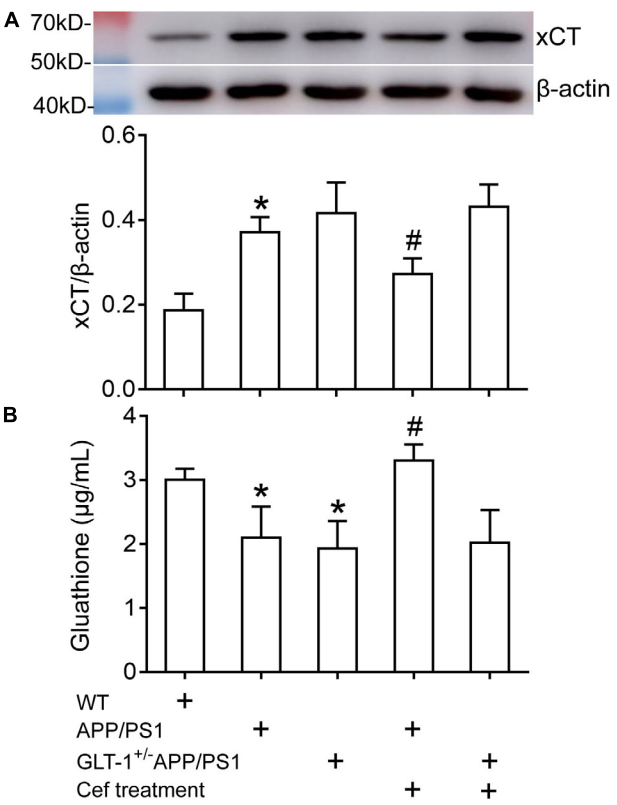

FIGURE 5 | The impact of Cef treatment on xCT expression and glutathione level in the hippocampus of APP/PS1 mice and GLT-$1^{\pm}$APP/PS1 mice assayed with Western blot analysis and glutathione assay kit. **(A)** Western blot analysis for xCT expression in which the top is the immunoreactive band of xCT and β-actin, the bottom bar graph is quantitative presentation of the immunoreactive bands by integral optical density. **(B)** Glutathione levels. *$p < 0.05$ vs. the wild-type group, #$p < 0.05$ vs. the APP/PS1 group, $n = 6$ per group. All the data were presented as mean ± standard deviation (SD). xCT expression increased, while glutathione level decreased in APP/PS1 and GLT-$1^{\pm}$APP/PS1 groups compared with the wild-type group. Cef treatment restored xCT expression and glutathione level in the APP/PS1 group, but had no effect in the GLT-$1^{\pm}$APP/PS1 group.

DISCUSSION

Glutamate transporter-1 is an important molecule in regulating neurotransmission mediated by glutamate, maintaining the appropriate concentration of glutamate and limiting excitotoxic effect of glutamate and neurodegeneration. GLT-1 loss or dysfunction has been implicated in the pathogenesis of AD. For example, postmortem investigation of patients with AD showed that GLT-1 was expressed more in activated astrocytes, and cognitive functions were preserved better before the patient's death (Kobayashi et al., 2018). Restoring GLT-1 expression by transgenic or pharmacological approaches in experimental animals significantly improved synaptic damage, Aβ deposition, and cognitive impairments (Takahashi et al., 2015; Mi et al., 2018). This suggests that upregulation of GLT-1 expression and uptake for glutamate may be profitable for preserving cognition in AD patients or animal models. Cef has been revealed to selectively increase GLT-1 expression and uptake activity, and this upregulation has been suggested to be protective in many models of diseases, such as amyotrophic lateral sclerosis (Rothstein et al., 2005) and Parkinson's disease (Hsu et al., 2015; Hsieh et al., 2017). Furthermore, Cef can ameliorate tau pathological changes and cognitive impairment by promoting the glutamate transporters in 3xTg AD model (Zumkehr et al., 2015) and long-term potentiation impairment and paired-pulse responses in OKA-induced AD model (Hamidi et al., 2019). We have recently showed that Cef can stimulate GLT-1 expression and improve cognitive impairment in APP/PS1 AD mice (Fan et al., 2018). In the present study, we confirmed the upregulation of GLT-1 expression and improvement on cognitive impairments after treatment of Cef in APP/PS1 AD mice. These findings were in accordance with the previous reports and further proved the improving effect of Cef on cognitive impairment in AD mice and the implication of GLT-1 in the process.

Glutamate transporter-1 scavenges glutamate by glutamate uptake. Previous studies have shown that glutamate transporter mRNA splice variants reduce glutamate uptake in AD-related conditions (Scott et al., 2011). Astrocytes derived from the cortex of patients with AD show a decreased glutamate uptake (Liang et al., 2002). Soluble Aβ could reduce glutamate uptake by 70% in crude synaptosomes of the hippocampus (Li et al., 2009). Overexpressions of mutant APP in the brain of transgenic mice reduce glutamate uptake (Li et al., 1997; Masliah et al., 2000). These results suggest that decreased glutamate uptake activity is involved in the pathogenesis of AD and correlates to cognitive decline (Masliah et al., 1996). Therefore, it is necessary to study the changes in GLT-1 uptake to elucidate the protective mechanism of Cef on cognitive deficits of AD. Thus, we measured glutamate uptake capacity with ^{3}H-glutamate in the present study. We found that, accompanied with downregulation in GLT-1 expression, glutamate uptake capacity of GLT-1 significantly declined in APP/PS1 AD mice, and Cef treatment clearly inhibited the decline. These results suggested that the decline in GLT-1 uptake activity is involved in the cognitive impairment of AD mice, and Cef may alleviate cognitive impairment by improving the activity of GLT-1 uptake.

However, in contrast to the improvement of Cef on the cognitive impairment of AD models, there were inconsistent reports in different studies related to the roles of Cef on cognition. For example, chronic administration of Cef in rats induced significant memory impairment in the novel object-recognition test (Matos-Ocasio et al., 2014). In addition, Cef disrupted motor skill learning and the functional outcome following focal ischemic cortical lesions (Kim and Jones, 2013). The detrimental effect of Cef in learning and memory might be associated with differences in animal models or administration approaches. It is necessary to design experiments to observe the influence of inhibiting or blocking GLT-1 expression and/or function to prove the effect induced by GLT-1. Previous work from our laboratory has shown that GLT-1 antisense oligodeoxynucleotides or dihydrokainic acid, a specific inhibitor of GLT-1, inhibited the beneficial effects of GLT-1 in global brain ischemic rats (Cui et al., 2015; Hu et al., 2015). Recently, we revealed that dihydrokainic acid inhibited the improvement of Cef on cognitive impairment in APP/PS1 mice (Fan et al., 2018). To more convincingly prove the improvement of Cef on cognitive impairment of APP/PS1 AD model mice and roles of GLT-1 in the improvement, we constructed partial GLT-1 knockout APP/PS1 mice in the present study. This mouse has phenotypes of APP/PS1 mice with downregulated GLT-1 expression. A previous study has reported that this mouse has more deteriorated impairment in cognitive functions and, thus, is a good animal model for the study to prove the involvement of GLT-1 in cognitive deficits of AD (Mookherjee et al., 2011).

Using this mouse in the present study, we showed that GLT-1 protein was significantly decreased, and Cef treatment had little upregulating effect on the downregulated GLT-1 expression in the GLT-1$^{\pm}$APP/PS1 mice. The results confirmed the effectiveness of the gene modification in knocking down GLT-1 expression. In addition, the knockdown of GLT-1 aggravated the cognitive dysfunction of the APP/PS1 mice in novel objective recognition and Morris water maze tests, which were consistent with a previous report (Mookherjee et al., 2011). The results suggested the involvement of GLT-1 impairment in the pathogenesis of AD. However, we are concerned whether GLT-1 loss inhibits the Cef-induced improvement of cognition. It was clearly shown that the improvement of Cef on cognitive impairment of APP/PS1 mice was inhibited after GLT-1 knockdown, i.e., the administration of Cef had no significant improvement on the cognitive impairment in GLT-1$^{\pm}$APP/PS1 mice. Simultaneously, the promoting impact of Cef on the activity of GLT-1 uptake in APP/PS1 mice was weakened in GLT-1$^{\pm}$APP/PS1 mice. The findings provide convincing evidence for the conclusion that Cef improves cognitive impairment of APP/PS1 AD mice by upregulating GLT-1 expression and uptake activity. Although it has been reported that GLT-1 knockdown might increase GLAST expression, another glutamate transporter (Pardo et al., 2006), whether other glutamate transporters and their cell type-specific expression, and glutamate receptors, including ionotropic and metabotropic glutamate receptors, are impacted after GLT-1 knockdown, and whether these influences participate in the cognitive improvements after Cef treatment in APP/PS1 AD mouse remain to be clarified. The elucidation of these issues

would promote the understanding of the effects of GLT-1 knockdown and ceftriaxone treatment on APP/PS1 AD mouse.

In addition to GLT-1, xCT is functionally related to glutamate homeostasis and GLT-1 uptake activity. Another novelty of the present study is that changes in xCT expression before and after Cef treatment in APP/PS1 and GLT-1$^{\pm}$APP/PS1 AD mice has been observed. We found that xCT expression was upregulated in APP/PS1 and GLT-1$^{\pm}$APP/PS1 mice. This finding is supported by previous reports. For instance, xCT expression in the cerebral cortex of adult Aβ PP23 mice increased, accompanied with a decreased glutamate uptake and increased extracellular glutamate (Schallier et al., 2011). Aβ injection increased xCT gene expression in the hippocampus of adult mouse (Qin et al., 2006). Amyloid precursor protein evoked glutamate export from xCT, which led to synaptic degeneration and neuronal death (Barger and Basile, 2001). These results indicated that xCT is involved in the pathological progression of AD. We think that increased xCT expression might be a compensatory change in the impairment of GLT-1 expression and uptake activity in APP/PS1 and GLT-1$^{\pm}$APP/PS1 mice. In our previous study, we have shown the downregulation of GLT-1 expression in APP/PS1 mice (Fan et al., 2018), and in the present study, we have shown a decline in the uptake activity of GLT-1. As the main function of GLT-1 is the uptake of glutamate, the decrease in GLT-1 expression and uptake activity leads to the accumulation of glutamate and an increase in extracellular glutamate concentration. Since glutamate is a competitive inhibitor of glutamate export of xCT, excess accumulation of glutamate in the extracellular space impedes the xCT activity, leading to decreases in the export of glutamate and then the import of cystine (Murphy et al., 1989; Patel et al., 2004). Consequently, xCT expression increases to compensate the impaired transporting activity and maintain normal transporting activity for glutamate and cystine. It has been reported that xCT expression increases under conditions of glutamate oxidative stress and GSH depletion (Lewerenz et al., 2006, 2013), which support the explanation.

With changes in xCT expression, the glutathione level in APP/PS1 mice decreased in comparison with that in wild-type mice. This decrease might result from oxidative stress induced by the excitotoxicity of glutamate and accumulation of Aβ in AD mice, which consume a lot of glutathione. Another factor causing the decrease in glutathione in AD mice might be associated with dysfunctions in the transporting activity of xCT, resulting from the accumulation of glutamate and increased extracellular glutamate concentration induced by the dysfunction of GLT-1 uptake activity mentioned above. It seems conflicting that xCT expression was upregulated, while glutathione content decreased in APP/PS1 mice. We think that this might result from the incomplete compensatory role of xCT expression and the following transporting activity. It means that the compensatory upregulation of xCT expression could not completely compensate the overdepletion and insufficient synthesis of glutathione resulting from oxidative stress and disturbed xCT transporting activity for cystine, respectively. Notably, Cef treatment restored the glutathione level to about sham level in the present study, and this effect was inhibited by GLT-1 knockdown in APP/PS1 mice, which suggested that Cef can regulate xCT activity by improving GLT-1 uptake activity. This result is consistent with a previous report that Cef can increase glutathione levels by activating transcription factor Nrf2 (Lewerenz et al., 2009) and is particularly valuable to increase the antioxidant capability and beneficial effects in diseases, such as AD.

Several studies reported that Cef increases xCT expression in fibroblasts and hippocampal cell line HT22 (Lewerenz et al., 2009, 2013), and in the nucleus accumbens core in rat modeled reinstatement of cocaine seeking (LaCrosse et al., 2017). In the present study, we found that upregulation of xCT expression in APP/PS1 AD mice was restored after Cef treatment, which means that the xCT expression in Cef-treated APP/PS1 AD mice was downregulated, rather than upregulated, compared with no Cef-treated APP/PS1 AD mice. This result seems in conflict with previous reports mentioned above. We believe that the change in xCT expression after Cef treatment in APP/PS1 AD mice might be, at least partly, a secondary change following GLT-1 upregulation induced by Cef treatment (**Supplementary Material**). It has been shown that excess glutamate in the extracellular space is a competitive inhibitor of xCT activity (Murphy et al., 1989; Patel et al., 2004). The upregulated GLT-1 expression and glutamate uptake resulting from Cef treatment decreased the extracellular glutamate concentration, which eliminated the inhibition of xCT activity due to the high extracellular glutamate concentration, and thus, xCT expression was restored in the APP/PS1 mice. Notably, that GLT-1 knockdown in APP/PS1 mice prevented Cef from restoring xCT expression and glutathione levels further proved the above assumption. Although we could not show upregulation in xCT expression after Cef treatment in APP/PS1 mice, our data are in agreement with that Cef can play a supporting effect for xCT activity by stimulating GLT-1 expression and uptake for glutamate, which promotes glutamate uptake of astrocytes and then supplies intracellular and reduces extracellular glutamate, respectively. This supporting effect promotes the activity of xCT, drives more cystine import to astrocytes, and increases synthesis of glutathione (Rimaniol et al., 2001; Lewerenz et al., 2006).

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are included in the article, further information is available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Hebei Medical University.

AUTHOR CONTRIBUTIONS

JG conducted most parts of the experiments and wrote the manuscript. LZL, SF, and LRL participated in parts of the experiments. CL and SL supplied the animals of the GLT-1

knockdown. WBL and XHX designed the study, performed the data analysis and interpretation, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.580772/full#supplementary-material

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The Effects of Nordic Walking With Poles With an Integrated Resistance Shock Absorber on Cognitive Abilities and Cardiopulmonary Efficiency in Postmenopausal Women

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Late adulthood is associated with atrophy of brain areas, which contribute to cognitive deterioration and increase the risk of depression. On the other hand, aerobic exercise can improve learning and memory function, ameliorate mood, and prevent neurodegenerative changes. This study demonstrates the effect of Nordic walking (NW) and NW with poles with an integrated resistance shock absorber (NW with RSA) on aerobic capacity and body composition in postmenopausal women. It also measures the brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) serum levels and determines correlations with cognitive functions and depression symptoms. These relationships with the use of NW with RSA as a new form of exercise have not been described thus far. In this study, 31 women (NW – 16, NW with RSA – 15) participated in eight weeks of training. The findings showed that only NW with RSA training caused a significant decrease in body mass and body mass index ($p < 0.05$). There were no significant changes in GDNF levels between groups studied. Regarding BDNF, a significant decrease ($p < 0.05$) in the NW group and an increase (not statistically significant) in the NW with RSA group was found. A comparative analysis of cognitive and depression outcomes and changes in BDNF and GDNF concentration showed no significant differences in the efficacy of either form of training. Training loads resulted in a significant increase in VO_2max in both the NW ($p < 0.01$) and NW with RSA ($p < 0.05$) groups. This indicates an improvement in cardiopulmonary efficiency of the examined women.

Keywords: walking training, neurotrophins, maximal oxygen uptake, Beck Depression Inventory, Stroop test

INTRODUCTION

In the aging process, a series of changes occur in the structures and systems of the human body. Functional changes associated with atrophy of certain areas of the brain, especially the hippocampus and prefrontal and temporal cortices, can lead to age-related cognitive decline (Raz et al., 2005; Driscoll et al., 2009; Kennedy et al., 2009), risk of depression (Steffens et al., 2000; O'Shea et al., 2018; Szymkowicz et al., 2019) and neurodegenerative diseases (Wilson et al., 2002). There is evidence to suggest that these changes are correlated with reduced levels of brain-derived neurotrophic factor (BDNF) (Tapia-Arancibia et al., 2008; Erickson et al., 2012) and glial cell line-derived neurotrophic factor (GDNF) (Wang et al., 2011; Sharma et al., 2016; Tsybko et al., 2017) in older adults and rodents. BDNF and GDNF belong to the family of trophic factors identified in the nervous system (Siamilis et al., 2009; Ghanbarzadeh et al., 2016) and various non-neuronal tissues, including skeletal muscles (Dupont-Versteegden et al., 2004), especially in response to physical exercise. BDNF regulates synaptogenesis and survival of adult neurons, enhances the mechanism of synaptic plasticity, thereby influencing cognition, and preventing depression and Alzheimer's disease (Weinstein et al., 2014; Ferrer et al., 2019; Ng et al., 2019). The main function of GDNF is to exert a protective effect on dopaminergic and cortical neurons, and spinal motoneurons, consequently improving motor function and thus preventing Parkinson's disease (Zigmond et al., 2009; Lau et al., 2011; McCullough et al., 2013). The important contribution of GDNF in the pathophysiology of neuropsychiatric disorders is also reported (Chu et al., 2018).

Human and animal studies have shown that physical activity can stimulate synthesis and the release of endogenous neurotrophins. The number of publications concerning the beneficial effect of various types of aerobic exercise and training on enhancing BDNF levels in the brain and peripheral blood both in animal models (Afzalpour et al., 2015; Eldomiaty et al., 2017; TaheriChadorneshin et al., 2017; Algaidi et al., 2019) and humans (Currie et al., 2009a; Damirchi et al., 2014; Huang et al., 2014; Dinoff et al., 2017) is constantly increasing. Training-induced increase in BDNF levels improves cognitive function (Best et al., 2015; Hákansson et al., 2017; Nilsson et al., 2020) and mood (Rethorst et al., 2009) in healthy people and people with cognitive impairment (Nascimento et al., 2015) and depression (Schuch et al., 2016; Kering et al., 2017). The animal studies confirm observations that training exerts a beneficial effect on learning and memory and ameliorates depression-like behavior (Lin et al., 2012; Marlatt et al., 2012; Lu et al., 2014).

In light of numerous BDNF studies, there is little information concerning the influence of exercise training on GDNF levels in humans (Roh and So, 2017). More extensive studies in animals, mainly rodents, provide data on GDNF levels in the hippocampus of rats exposed to stress (Jiang et al., 2014), in the striatum of hemiparkinsonian mice (Speck et al., 2019) and the spinal cord and skeletal muscles (McCullough et al., 2013; Vianney et al., 2013) in response to physical activity.

There are several lines of evidence to indicate that physical exercise not only can generally slow down aging and prevent chronic, psychiatric and neurodegenerative diseases, but is very important in maintaining normal brain function or softening its progressive loss (Haskell et al., 2007; Pedersen and Saltin, 2015; Tan et al., 2017). This issue is especially important in women because of the gender differences associated with aging, namely the consequences of menopause. A decrease in estrogen production and corresponding estrogen deficiency leads to a decline in physical functions and negatively influences cognition and mood, and contributes to the development of neurodegenerative processes (Li et al., 2014). Lack of neuroprotective action of estrogen in postmenopausal women causes their risk of Alzheimer's disease to be higher when compared to age-matched men (Vina and Lloret, 2010).

In the above context, this investigation was undertaken to demonstrate the impact of walking training, i.e., Nordic walking (NW) with classic poles and NW with poles with an integrated resistance shock absorber (NW with RSA), on BDNF and GDNF serum levels, and correlations with cognitive functions and depression levels in postmenopausal women. The new form of NW is training with modified poles, which allows combining aerobic and strength training. These modified poles contain a built-in RSA. An elastic tape between two permanent elements in RSA poles allows additional resistance to be obtained by increasing the overall intensity of exercise and calorie consumption. Literature analysis indicates that this is the first study to describe these relationships.

MATERIALS AND METHODS

Study Design and Participants

Initially, 50 women aged 60–75 were recruited for the study. Subjects were recruited by advertisements in local media and at informative events and were qualified to participate in the project based on medical history and cardiology tests. The following exclusion criteria have been applied (presence of at least one of the factors listed below): diseases of the locomotor system preventing independent movement, giant obesity, active or post cancerous disease (ongoing radiation or chemotherapy treatment), liver diseases (ALT $>$ 3$\times$ borderline) except for liver disease, chronic kidney disease (eGFR $<$ 30 mL/1.73 m^2/min), acute inflammation (CRP $>$ 5 mg/dL), unstable ischemic heart disease, after an ischemic or hemorrhagic stroke ($<$6 months), post-STEMI (ST-elevation myocardial infarction) women with a drug-eluting stent implantation, NSTEMI (non-ST-elevation myocardial infarction) ($<$12 months), inherited metabolic disorders (phenylketonuria and galactosamia), autoimmune diseases (an acute thyroiditis, celiac disease, systemic connective tissue disease, hemolytic anemia, vitiligo, Addison's disease, hyperbilirubinemia), non-specific enteritis (Crohn's disease and ulcerative colitis), psychological disorders, antibiotic therapy, steroid therapy (ongoing), drug, and alcohol addiction (a daily consumption of more than one portion of alcohol). Finally, 40 women (mean age 66.64 years, SD = 4.20) were included in the project and were randomly assigned to two groups with a

different training program. Randomization was performed as simple random allocation; each subject identifier was forwarded to a person who was not involved with the conduct of the study, and who performed the randomization blindly using a computer list. All subjects were of Caucasian origin, in particular, a native Polish population from the Great Poland region. The subjects were asked not to change their eating habits for the duration of the project and not to perform any additional physical activity, except for the one carried out in the research project. Five women withdrew from the project, while four were excluded due to performing additional physical activity. Thus, the results of 31 people (NW – 16, NW with RSA – 15) were statistically analyzed. An information sheet was provided to each woman approached to participate in the study and on agreement to participate, informed written consent was obtained. The entire study was conducted over nine weeks (from the 13th of February 2019 until the 17th of April 2019).

Training Program

The research experiment was held twice a week for 8 weeks (16 training sessions). Training for both groups was performed at the same time. Women from the NW group used classic poles, while the NW with RSA group used poles with an integrated RSA having an elastic resistance of 4 kg (Slimline Bungy Pump, Sports Progress International AB, Sweden). Each training session started with 10–15 min of warm-up. After each half of the planned distance (about 1.7–2.2 km at a rate of about 1 km per 10 min) participants of the classes performed strength exercises and balance training (15 min). After the rest of the planned distance, stretching exercises took place at the end of the training (15 min). During the meetings, the walking distance from 3.5 to 4.5 km, as well as the number of exercises performed from 8 to 12 repetitions was gradually increased. Exercise intensity corresponded to 50% HRR (heart rate recovery) during 1–8 training sessions, while from sessions 9–16 the intensity increased to 65–70% HRR and was monitored based on heart rate (HR) (Polar Electro Oy, Kernpele, Finland) (Marciniak et al., 2020). The minimum required presence at 13 training sessions was adopted (80%). The trainer conducting the classes had appropriate qualifications. The training plan included American College of Sports Medicine (ACSM) recommendations for adults and healthy older people: one set of 8–10 exercises for major muscle groups 2–3 days per week; most exercisers should perform 8–12 repetitions of each exercise.

Blood Analysis

Testing sessions were scheduled 4 days before the training period and 4 days after the final training bout. Subjects were instructed to abstain from physical exercise between the final training bout and the final test. Participants reported to the laboratory between 07.00 and 8.00 h after an overnight fast. The time of day was held constant for every subject (±30 min). Blood samples (10 mL) were taken from the ulnar vein after a resting period of at least 10 min using a S-Monovette syringe tube (SARSTEDT, Germany), then placed in tubes containing a clot activator, and centrifuged (1500 × *g*, 4°C, 4 min) (Universal 320R; Hettich Lab Technology, Tuttlingen, Germany) to separate the serum. The samples were frozen and stored at −80°C until the time the analyses were performed (U410, Ultra-Low Temperature Freezer, New Brunswick Scientific, United States).

Serum BDNF levels were quantified using a commercially available ELISA (enzyme-linked immunosorbent assay) kit (Shanghai Sunred Biological Technology Co., Ltd.). The concentrations of GDNF were measured with an ELISA kit (Cloud-Clone Corp., Katy, TX, United States). The sensitivities of the ELISA kits were as follows: 0.05 and 0.058 ng/mL, respectively. The intra- and inter-assay coefficients of variation (CVs) were less than 10 and 12%, respectively. The samples were read using a Synergy 2 SIAFRT multi-detection microplate reader (BioTek, United States) at the manufacturer's recommended wavelength.

Exercise Test

Aerobic capacity was assessed with the modified Astrand–Rhyming protocol for predicting VO_2max by an ergometer Kettler DX1 Pro, (Ense – Parsit, Germany) and HR was monitored using a Polar A-5 pulse meter (Polar Electro Oy, Kernpele, Finland) (Gillett, 1993). The predicted VO_2max was read from the nomogram (Astrand and Ryhming, 1954) or accompanying tables (Astrand and Ryhming, 1954) and multiplied by both the Astrand and the von Dobeln age correction factors. These two predictions in L/min were then converted to mL/kg/min.

Body Composition

Bioelectric impedance analysis (BIA) is commonly used in field surveys and also as a supplement to conventional anthropometry (Heyward and Wagner, 2004). The body composition of women was estimated with BIA using the TANITA MC-980MA (TANITA, Japan), following the directions and procedures of the manufacturer. The unit provides a profile for the individual including weight, % fat, % fat-free mass, muscle mass, and visceral fat tissue. The women stood erect holding the hand electrodes and with bare feet on the contact electrodes of the BIA unit. Waist circumference was measured halfway between the lower rib and the iliac crest in a horizontal plane. Hip circumference was measured as the widest horizontal circumference over the buttocks.

Psychological Examination

Depressive symptoms were assessed with the Beck Depression Inventory (BDI). The BDI is a self-reported measure of depression consisting of 21 items covered to detect the total score for measuring the symptoms of depression (Beck et al., 1988). Each item is scored with 0–3 points. The items are summed and the total possible score for the scale ranges from 0 to 63, with higher values indicating greater depression levels. It has proven to be sensitive to exercise-induced changes in healthy adults and postmenopausal women without depression (Bernard et al., 2015).

The Stroop Color and Word Interference Test was developed to measure selective attention and cognitive flexibility (Stroop, 1935). It refers to the impairment of the reading speed and color recognition due to interfering information and consists of two

parts. In the first part, Reading Color Names in Black (RCNb), the subject is required to read 10 rows of five words for color names written in black on a white sheet as soon as possible. In the second part, Naming Color of Word/different (NCWd), subjects should name the individual font color as soon as possible, where the font color of the word is different from the color that is written. Change of reaction form is required (switching from content to color). The time in seconds needed to make the first and second parts is estimated (Homack and Riccio, 2004).

The Trail Making Test (TMT) is a widely used neuropsychological test of visual attention and task switching (Salthouse, 2011). It consists of two parts in which the subject is instructed to connect a set of 25 dots as quickly as possible while still maintaining accuracy. If the subject makes an error, the test administrator corrects them before the subject moves on to the next dot (Bowie and Harvey, 2006). In the first part, the targets are all numbers (1, 2, 3, etc.) and the test taker needs to connect them in sequential order as fast as possible. The first part is used primarily to examine cognitive processing speed. In the second part, the participant is to draw lines to connect circled numbers and letters in an alternating numeric and alphabetic sequence (i.e., 1-A-2-B, etc.) as fast as possible. This part is used to examine executive functioning (Tombaugh, 2004; Bowie and Harvey, 2006). The goal of the test is to finish both parts as quickly as possible, with the time taken to complete the test being used as the primary performance metric.

The Verbal Fluency Test (VFT) is one of the best known and useful neuropsychological tools based on the language functions diagnosis. VFT allows one to discover disorders within the cognitive sphere, including evaluating many functions such as verbal fluency, processes of attention, information processing rate, working memory, and executive functions (Ruff et al., 1997). There are two versions of VFT: using the formal (letter-related) criterion (i.e., phonemic fluency) and semantic (category-related) criterion (i.e., semantic fluency). In the formal version of VFT the respondent generates the highest possible number of words beginning with a given letter. The semantic fluency test consists of listing, by the examiner, the highest possible number of words belonging to a certain category (in our case "fruit"). We used the letter "P." Each task is performed within 1 min (Ponichtera-Kasprzykowska and Sobów, 2014).

Statistical Analyses

The obtained results were analyzed statistically using the Dell Statistica data analysis software system (version 13, software.dell.com, Dell Inc., Round Rock, TX, United States). The sample size was calculated based on data from study of Zoladz et al. (2014) that determined the average level of plasma BDNF. In addition, this study shows methodological similarities with the present study (the 8-weeks NW training and age). After calculations adopting a power as 1-beta error probability: 95%, effect size: 0.80, and error assumed as alpha: 0.03 (two-sided), eight participants were assigned to allocate equally into each group (four NW and four NW with RSA). Therefore, the present study was initiated with 40 women divided randomly between the NW ($n = 20$) and NW with RSA ($n = 20$) groups. The data are presented as mean and standard deviation (SD). The normality of distributions was verified using the Shapiro–Wilk test. It was assumed that the analysis of repeated-measures ANOVA 2 × 2 (time × group) will be performed if the differences in variables over time and between groups are significant. The Mann–Whitney U test and the Wilcoxon test were employed for non-normally distributed variables, respectively, to evaluate the significance of differences between the groups and test dates. Spearman's rank analysis was used to calculate correlation coefficients. The level of statistical significance was set at $p \leq 0.05$. Effect sizes (ESs) were calculated as the difference between means divided by the pooled SD. Using Cohen's (1988) criteria, an effect size $\geq$0.20 and $<$0.50 was considered small, $\geq$0.50 and $<$0.80 medium, and $\geq$0.80 large.

RESULTS

Comparative analysis of somatic characteristics, cardiopulmonary efficiency, and blood BDNF and GDNF levels measured before and after an 8-week NW and NW with RSA training program is presented in **Table 1**. The 8-week training program caused a significant decrease in waist circumference of subjects in both NW [$p < 0.05$ (ES: 0.13)] and NW with RSA [$p < 0.01$ (ES: 0.27)] training groups. There was no significant difference in post-workout change in waist circumference between the two groups. NW with RSA training caused a significant decrease in body mass [$p < 0.05$ (ES: 0.09)] and BMI [$p < 0.05$ (ES: 0.10)]. Statistical analysis showed a significant difference in the change of the described indicators between the examined groups. Training loads implemented in the project resulted in a significant increase in cardiorespiratory efficiency in the NW [$p < 0.01$ (ES: 0.93)] and NW with RSA [$p < 0.05$ (ES: 0.54)] groups. There was no significant difference in post-workout change in cardiorespiratory efficiency between the two groups.

In both NW and NW with RSA groups, a slight but not significant increase in blood GDNF concentrations was revealed. However, there were no significant changes in blood GDNF levels between groups studied. In the group covered by traditional NW training, a significant decrease in BDNF blood concentration was found [$p < 0.05$ (ES: 0.11)]. The opposite was noted in the NW with RSA group. However, this change was not statistically significant.

A comparative analysis of cognitive and mental health outcomes in response to the intervention program is shown in **Table 2**. The 16 training sessions of NW caused no significant changes in the cognitive and mental health outcomes. In the case of variables such as depression, naming interference, and executive function, expected positive changes were observed, but they were not statistically significant. Similarly, under the influence of NW with RSA training no significant changes were noted in most of the tested parameters. However, in the naming interference, statistically significant positive changes as an effect of the applied exercise program were noted [$p < 0.05$ (ES: 0.55)]. Statistical analysis indicated that no significant differences in post-workout changes results were present between the two groups studied (NW and NW with RSA).

TABLE 1 | Somatic characteristics, cardiopulmonary efficiency, and biochemical indices of women subjected to an 8-week NW and NW with RSA training program.

	NW ($n = 16$) (M ± SD)			NW with RSA ($n = 15$) (M ± SD)		
	Baseline	**9 weeks**	**Change**	**Baseline**	**9 weeks**	**Change**
Age, years	66.38 ± 3.74			68.00 ± 4.33		
Body height, cm	159.80 ± 5.09			161.91 ± 3.62		
Body mass, kg	67.70 ± 9.99	67.59 ± 10.21	−0.11 ± 0.75	73.77 ± 10.47	72.81 ± 10.14*[a]	−0.97 ± 1.38#[a]
BMI, kg/m^2	26.44 ± 3.02	26.39 ± 3.03	−0.07 ± 0.31	28.16 ± 3.95	27.78 ± 3.76*[a]	−0.38 ± 0.54#[a]
FAT, %	35.09 ± 4.37	35.32 ± 3.88	0.16 ± 1.43	37.75 ± 4.84	37.52 ± 4.94	−0.23 ± 1.21
FFM, %	43.19 ± 5.29	43.21 ± 5.73	0.04 ± 1.17	45.47 ± 4.96	45.05 ± 4.14	−0.42 ± 1.14
SMM, kg	40.97 ± 5.02	41.01 ± 5.47	0.06 ± 1.12	43.15 ± 4.25	42.77 ± 3.92	−0.38 ± 1.09
VFA	9.07 ± 1.53	9.07 ± 1.53	0.00 ± 0.00	10.15 ± 1.86	10.08 ± 2.06	−0.08 ± 0.64
Waist circumference, cm	83.10 ± 8.63	82.02 ± 8.19*[a]	−1.08 ± 1.79	88.50 ± 9.88	85.93 ± 9.19*[b]	2.57 ± 3.05
Hip circumference, cm	99.44 ± 7.00	99.31 ± 6.93	−0.13 ± 9.93	104.43 ± 5.81	104.00 ± 6.31	−0.43 ± 2.69
VO_2max, mL/kg/min	27.68 ± 4.89	32.14 ± 4.79*[b]	4.45 ± 4.38	28.05 ± 4.45	30.45 ± 4.49*[a]	2.40 ± 3.87
BDNF, ng/mL	9.29 ± 15.48	7.76 ± 13.68*[a]	−1.53 ± 5.04	9.52 ± 11.44	9.78 ± 12.79	0.26 ± 5.36
GDNF, ng/mL	1.69 ± 0.88	1.86 ± 0.85	0.17 ± 0.76	1.67 ± 1.00	1.85 ± 1.13	0.18 ± 0.68

Values are means (± SD).
BMI, body mass index; FAT, body fat mass; FFM, body free fat mass; SMM, skeletal muscle mass; VFA, visceral fat area; VO_2max, maximal oxygen uptake; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor.
[a]$p < 0.05$.
[b]$p < 0.01$.
**Level of significance of change between baseline and after nine weeks for each group*
#Level of significance of post-workout changes in the studied indicators between the examined groups.

TABLE 2 | Cognitive and mental health outcomes in response to the intervention program.

	NW ($n = 16$) (M ± SD)			NW with RSA ($n = 15$) (M ± SD)		
	Baseline	**9 weeks**	**Change**	**Baseline**	**9 weeks**	**Change**
BDI (points)	9.1 ± 8.27	8.3 ± 6.74	−0.8 ± 4.18	8.33 ± 7.02	7.2 ± 6.26	−1.1 ± 5.18
STROOP 1 (s)	26.1 ± 2.46	26 ± 2.89	−0.1 ± 2.35	24.8 ± 3.99	24.9 ± 3.78	0.14 ± 2.39
STROOP 2 (s)	68.7 ± 11.78	65.6 ± 13.43	−3.1 ± 9.45	69.9 ± 13.63	62.9 ± 11.54*[a]	−7.0 ± 9.79
TMT A (s)	35.9 ± 10.77	33.8 ± 9.7	−2 ± 9.55	34.0 ± 4.31	37.0 ± 10.54	2.97 ± 9.44
TMT B (s)	84.4 ± 31.84	77.3 ± 29.6	−7.2 ± 27.52	94.8 ± 39.71	92.1 ± 48.59	−2.7 ± 23.13
VF L (words)	17.7 ± 6.38	16.9 ± 4.49	−0.8 ± 4.96	15.1 ± 4.61	14.8 ± 6.25	−0.2 ± 3.61
VF S (words)	14.7 ± 2.44	15.4 ± 2.03	0.7 ± 3	14.9 ± 3.03	15.4 ± 3.64	0.53 ± 2.83

Values are means (±SD).
BDI, Beck's Depression Inventory; STROOP 1, reading interference; STROOP 2, naming interference; TMT A, cognitive processing speed; TMT B, executive function; VF L, formal fluency (letter-related criterion); VF S, semantic fluency (category-related criterion).
[a]$p < 0.05$.
**Level of significance of the change between baseline and after 9 weeks for each group.*

The findings obtained in this study indicate some relationships between variable changes before and after an 8-week training program. However, at the beginning it should be emphasized that no correlation was found between post-workout changes in cardiopulmonary efficiency and changes in BDNF and GDNF blood levels in either of the NW and NW with RSA groups.

Significant correlations between BDNF concentrations and certain cognitive and mental health outcomes were noted among respondents from both study groups (NW and NW with RSA). In the NW group, higher BDNF concentrations resulted in better early TMT B results ($r = -0.51$; $p = 0.045$). In addition, changes in BDNF levels correlated with changes in TMT B results (executive function) in this group ($r = -0.67$; $p = 0.004$). Moreover, women with higher GDNF levels at baseline, achieved better results in the VF S test ($r = 0.61$; $p = 0.012$). We observed correlations between GDNF concentrations and VF L test results after the NW training program ($r = -0.54$; $p = 0.032$). Furthermore, changes in GDNF levels positively correlated with changes in VF S ($r = 0.77$; $p = 0.001$). There was also a negative correlation between the post-workout change in body mass, BMI, FFM, SMM, and the change in resting GDNF concentrations in the blood ($p < 0.05$). In the NW with RSA group, the higher the BDNF concentration, the better the TMT A test results were at baseline ($r = -0.55$; $p = 0.034$). BDNF concentrations were also found to correlate significantly with the results from the VF L test both at the beginning ($r = 0.56$; $p = 0.031$) and at the end of experiment ($r = 0.65$; $p = 0.008$). Additionally, a correlation between

BDNF concentration and VF S test results was also recognized in the NW with RSA group ($r = 0.70$; $p = 0.004$) after the experiment was completed.

Statistical analysis showed that there were no significant correlations between GDNF concentrations and the results obtained in the field of cognitive and mental health in the NW with RSA group. Moreover, in the group of women included in the NW with RSA training program, a significant relationship was observed regarding the post-workout change in waist circumference and change in BDNF concentration ($r = -0.71$; $p = 0.003$) in addition to GDNF concentration ($r = 0.61$; $p = 0.015$). Also, analysis of post-workout changes in the levels of cardiopulmonary efficiency showed correlations with the changes in TMT A ($r = -0.53$; $p < 0.05$).

DISCUSSION

In this study in postmenopausal women, BDNF and GDNF concentrations in the blood were investigated along with cognitive functions and depressive symptoms to evaluate the effects of two types of walking training, i.e., NW with classic poles and NW with RSA using poles equipped with a system that provides resistance whenever the pole is pressed down. Relationships with cardiopulmonary efficiency and body composition were also analyzed. The main result presented herein is that NW compared to NW with RSA is a more effective form of walking training for postmenopausal women.

There is a lot of research on the effectiveness of the NW activity in reducing body weight, improving cardiopulmonary efficiency, glucose, and lipid metabolism in postmenopausal women (Latosik et al., 2014; Hagner-Derengowska et al., 2015). It has also been shown to have a beneficial effect on improving cognitive function, mental health, and slowing down neurodegenerative changes associated with aging (Gmiąt et al., 2018).

Regarding NW with RSA, there are currently no published scientific reports on the effects of this relatively new kind of training on neurotrophins level, cognitive function, and depressive symptoms in people of all ages. However, there is one available study concerning the effects of the NW with RSA training on the functional fitness of older women. The authors indicated that in comparison to NW, the NW with RSABP training provides additional resistance effort during marching, improved muscle strength, and aerobic endurance as a result of increased muscle activation (Marciniak et al., 2020). According to information from the NW with RSA producer, the use of NW with RSA poles and NW poles provides an average of 21 and 18% higher oxygen consumption, respectively, compared to normal walking[1]. Thus, the intensity of NW with RSA training is higher causing it to have a greater impact on the body.

A comparative analysis of both forms of walking used in the present study revealed that 16 training sessions of NW and NW with RSA did not cause significant changes in the cognitive and mental health outcomes. For variables such as depression, naming interference, and executive function, expected positive changes were observed, but they were not significant. Statistical analysis showed that between NW and NW with RSA groups there were no differences in the scope of post-workout changes of examined indicators.

Many studies have been done to identify appropriate forms of exercise and training that prevent the decline of cognitive function (Lista and Sorrentino, 2010) and reduce the risk of depression (Yuenyongchaiwat et al., 2018) during aging. In response to physical activity, there is an increase in BDNF levels in the brain and peripheral blood (Kang et al., 2020). For GDNF, the available data in humans are scarce (Roh and So, 2017), and not many arise from animal studies (McCullough et al., 2013; Vianney et al., 2013; Jiang et al., 2014; Speck et al., 2019).

A meta-analysis based on 55 studies showed that 39% of them documented a significant increase in BDNF blood levels after acute exercise, and 61% of studies reported no post-workout change. Longer training units, regardless of the type of exercise performed, were more effective. Gender and baseline levels of physical fitness had a significant impact on post-workout BDNF changes (Dinoff et al., 2017).

On the other hand, there is evidence of a post-workout decrease in BDNF concentrations in response to diverse training programs (Correia et al., 2010; Walentukiewicz et al., 2018). In our study concerning post-workout changes in BDNF blood levels in the women examined, the different effects of training loads were demonstrated. In subjects from the NW group, there was a significant decrease in BDNF concentrations after 8 weeks of training. This finding corresponds well with that received after 12 weeks of NW training in elderly women (Walentukiewicz et al., 2018). Unlike the NW group, the upward trend in BDNF concentrations in the NW with RSA group observed in our study is difficult to explain, especially since no reports of this type of training are available.

Approximately three-quarters of BDNF production both at rest and during exercise occurs in the brain. Its decreased concentration in the periphery may result from greater use by skeletal muscle to regulate lipid metabolism in muscle fibers and absorption by the hippocampus and prefrontal cortex, which is pivotal for cognitive abilities (Rasmussen et al., 2009). Animal studies have shown that exercise causes an increase of BDNF uptake from the blood which is then involved in regeneration processes (Yu et al., 2017).

It should be noted that neither NW nor NW with RSA training used in the project caused an increase of GDNF concentration in the blood. Due to the lack of research on the impact of these forms of physical activity on GDNF concentration, the obtained results cannot be confirmed. However, there is a study on the effects of pharmacological treatment on GDNF levels in women with schizophrenia and depression (Skibinska et al., 2017).

[1] http://www.bungypump.se/

It is well known that high levels of endogenous neurotrophins, BDNF and GDNF, in the brain and peripheral blood reduce the hippocampus gray matter loss, improve memory, and reduce the occurrence of depression in the elderly (see section "Introduction"). The direction and size of changes in the concentration of neurotrophins is related to the duration of the training, its intensity, the type of exercises performed, and the subjects' health. Erickson et al. (2012) demonstrated the high effectiveness of moderate-intensity aerobic exercises performed by older people on the increase in BDNF concentration, hippocampus volume, and cardiorespiratory fitness compared to stretching exercises. Physical activity varying in intensity did not reveal changes in post-workout BDNF plasma concentrations in the elderly. Despite this, there was an observed increase in gray matter volume in the prefrontal cortex and beneficial effects on memory function independently of training intensity (Ruscheweyh et al., 2011). Exercises may also induce normalization of decreased BDNF serum concentration in elderly women with depression (Laske et al., 2010) and improve cognitive function and cardiorespiratory fitness in older adults with glucose metabolism disorders (Baker et al., 2010).

The NW is characterized by lower intensity of effort compared to NW with RSA. The metabolism of working muscles is dominated by a greater share of oxygen metabolism compared to NW with RSA walking at the same speed. The post-workout increase in cellular mitochondrial transformations is one of the factors responsible for increasing the VO_2max value. Our findings indicate an 86% greater increase in the VO_2max in women from the NW group (large effect size) in comparison to the NW with RSA group (medium effect size). The VO_2max value is largely determined by the cardiovascular, respiratory, and metabolic fitness of the muscle, and its post-workout growth is mainly caused by moderate-intensity efforts. High-intensity training not only does not increase the VO_2max value but is not suitable for older people (Coelho et al., 2013). The NW training leads to a decrease in body weight, body fat mass and BMI, increasing the level of aerobic capacity in people of all ages. The increase in maximum oxygen uptake results from an improvement in cardiovascular and respiratory efficiency, but also from an increase in the oxidative capacity of the muscles themselves. The improvement in aerobic capacity resulting from exercise training may be primarily a protective factor for the aging cardiovascular system and may positively affect the quality of life of the elderly (Figard-Fabre et al., 2010; Gram et al., 2010). On the other hand, the use of additional weights on the hands during NW training in the elderly does not increase energy expenditure, but only increases the activity of the muscles themselves. Due to the lack of physiological and biomechanical benefits, it is not recommended in the elderly (Schiffer et al., 2011). Strength training in the elderly significantly increases muscle mass and strength. Thus, it reduces difficulties in performing daily activities, increases energy expenditure and has a positive effect on body composition. Typical strength training leads to metabolic changes in the muscle and increase in its mass, without beneficial changes in the value of the VO_2max. Only a small combination of strength training with endurance training increases the cardiopulmonary efficiency without weakening the post-training increase in strength and muscle mass (Cadore et al., 2014). Studies conducted on young people show that exercises performed with an intensity of 70% VO_2max cause a greater increase in BDNF concentration than those with an intensity of 40% VO_2max (Kim and Kim, 2018). In turn, Currie et al. (2009b) revealed a negative correlation between BDNF concentration at rest and VO_2max value ($r = -0.35$; $p < 0.05$), and the level of physical activity ($r = -0.43$; $p < 0.01$). These results indicate that increased levels of cardiorespiratory fitness and high levels of physical activity are associated with lower concentrations of resting BDNF in the serum of healthy people (Currie et al., 2009b).

In our study, both NW and NW with RSA training lead to the improvement or maintenance of cognitive abilities and mental health as well as an increase in cardiopulmonary efficiency so immensely important to elderly people. However, with NW with RSA training, despite the greater effectiveness in reducing body weight, BMI, or waist circumference, the second period of the study showed a tendency to decrease muscle mass. Post-workout loss of muscle component may indicate the occurrence of overload changes and improper selection of training loads. Therefore, the loads applied in NW with RSA training seem to be too intense for postmenopausal women, and the classic NW seems to be more beneficial for this age group.

CONCLUSION

The recent and so far, the only investigation with the use of NW with RSA training concerns the assessment of functional fitness in older women (Marciniak et al., 2020). It has been demonstrated that additional resistance during NW with RSA causes increased muscle activation and, as a result, improved muscle strength and aerobic endurance in comparison to NW. Moreover, it has a significant impact on the level of physical fitness. In our study with the use of NW with RSA and NW, a comparative analysis of cognitive and depression outcomes and changes in blood concentrations of BDNF and GDNF showed no significant differences in the efficacy of either form of training. The significant increase in VO_2max observed in both forms of physical activity may be evidence of an improvement in cardiopulmonary efficiency. To the best of our knowledge, this is the first published report that compares the effectiveness of NW with RSA training and the traditional form of NW considering the relationships mentioned above.

Further research is needed that includes pre-menopausal women to compare the effects of NW and NW with RSA training on BDNF and GDNF levels, as well as on cognitive function, risk of depression, and cardiopulmonary efficiency. These studies would be aimed at developing effective walking training programs taking into account exercise variables such as

duration, volume, intensity, and resistance used in NW with RSA in different age groups both in healthy women and women with neuropsychiatric or metabolic diseases.

DATA AVAILABILITY STATEMENT

The data analyzed in this study are available on request to the corresponding author KD, domaszewska@awf.poznan.pl.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institute for Research in Biomedicine (IRB) at the Poznań University of Medical Sciences (2019-02-07; Ethics Approval Number: 245/19). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KD: conceptualization, methodology, writing – original draft preparation, data curation, formal analysis, resources, and project administration. MK and MW: data curation, resources, and writing – original draft preparation. KW: resources and funding acquisition. UC and KM: data curation and resources. DB writing – review and editing, data curation, resources, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Metabolomic and Lipidomic Profiling of Preoperative CSF in Elderly Hip Fracture Patients With Postoperative Delirium

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Objective: To investigate dysregulated molecules in preoperative cerebrospinal fluid (CSF) of elderly hip fracture patients with postoperative delirium (POD), in order to identify potential pathological mechanisms and biomarkers for pre-stage POD.

Materials and Methods: This nested case control study used untargeted metabolomic and lipidomic analysis to profile the preoperative CSF of patients (n = 40) who developed POD undergone hip fracture surgery (n = 10) and those who did not (n = 30). Thirty Non-POD patients were matched to 10 POD patients by age (± 2 years) and Mini Mental State Examination score (± 2 points). CSF was collected after successful spinal anesthesia and banked for subsequent analysis. On the first two postoperative days, patients were assessed twice daily using the Confusion Assessment Method-Chinese Revision. CSF samples from the two groups were analyzed to investigate possible relevant pathological mechanisms and identify candidate biomarkers.

Results: Demographic characteristics of the groups were matched. Eighteen metabolites and thirty-three lipids were dysregulated in the preoperative CSF of POD patients. Pathway enrichment analysis revealed perturbations in D-glutamine and D-glutamate metabolism; glycerophospholipid metabolism; alanine, aspartate and glutamate metabolism; sphingolipid metabolism; histidine metabolism; and arginine biosynthesis at the pre-delirium stage. Receiver operating characteristic curve analysis indicated that phosphatidylethanolamine (PE, 40:7e), with an area under the curve value of 0.92, is a potential biomarker for POD.

Conclusion: Multiple pathological mechanisms in the POD group were involved before surgery, including neuroinflammation, oxidative stress, and energy metabolism disorders induced by hypoxia, as well as neurotransmitter imbalances such as increased dopamine and glutamate, and decreased glutamine. These metabolic abnormalities potentially increase the fragility of the brain, thus contributing to POD. PE (40:7e) might

be a potential biomarker for POD. Not only do our results provide potential biomarkers for POD, but also provide information for deep pathological research.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier ChiCTR1900021533.

Keywords: cerebrospinal fluid, postoperative delirium, hip fracture, metabolomics, lipidomics

INTRODUCTION

Patients over 65 years of age are the largest consumers of procedural care, and postoperative delirium (POD) is one of the most common complications experienced by elderly patients during the postoperative period (Daiello et al., 2019). POD is an acute neuropsychiatric syndrome occurring in the hours to days after anesthesia and surgery (Vutskits and Xie, 2016), which can elicit durable deficits in executive function, memory, attention, and other cognitive domains (Eckenhoff et al., 2020). The incidence of POD is 20–45% among elderly adult surgery patients (Rudolph and Marcantonio, 2011; Inouye et al., 2014). As for hip fracture induced by traumatic stimulation in elderly patients, the incidence may reach as high as 53.3% (Aldecoa et al., 2017). POD primarily occurs 24–72 h after surgery, and most symptoms disappear in 1 week. However, it is linked with persistent impairments in brain function, including cognitive decline (Inouye et al., 2016), increased risk for Alzheimer's disease (AD) (Olofsson et al., 2018), and serious negative outcomes on patient prognosis such as longer hospitalization, decline in physical function, and even death (Schmitt et al., 2012). Considering the aggravation of an aging global population, the incidence of POD has become a major evaluation index of medical quality and safety (Berian et al., 2018).

As for POD patients, their preoperative brain functional reserve has already decreased, therefore, they have a higher risk to develop delirium after surgery. Thus, screening and providing greater attention to these potential higher-risk patients represents an important issue. The blood–brain barrier, which acts as a strict control point for what can enter the brain, is made up of tight junctions between endothelial cells lining blood vessels, astrocytic end feet, and a basement membrane. As an overwhelming majority of molecules indicative of central nervous system damage cannot be detected in the vasculature, cerebrospinal fluid (CSF) is the best body fluid to accurately demonstrate and evaluate biochemical changes following brain damage. Despite the prevalence and clinical significance of POD, its pathophysiology is still unclear. Clinical symptoms fluctuate and, at present, no reliable biomarkers have been identified. In view of this, identifying potential indicators for POD is an urgent clinical task. Evaluating dysfunctional metabolite expression in CSF using metabolomic and lipidomic analysis may enhance our understanding of pathological changes in POD patients at the molecular level.

We hypothesized that differentially expressed metabolites and lipids in preoperative CSF are associated with POD in elderly orthopedic patients. Our findings provide valuable scientific clues for the investigation of POD neuropathogenesis and facilitate more specific biomarker studies in the field.

MATERIALS AND METHODS

Patients and Setting

This study was approved by the Beijing Jishuitan Hospital Medical Science Research Ethics Committee (JLKS201901-04) and conformed to the principles of the Declaration of Helsinki. CSF samples were obtained for the purpose of laboratory research. All methods were performed in accordance with relevant guidelines and regulations. With written informed consent, this study was registered at the Chinese Clinical Trial Registry (ChiCTR1900021533). All participants were recruited from Beijing Jishuitan Hospital (Beijing, China) from March 2019 to August 2019. Eligible patients were at least 65 years of age with acute hip fracture injury (no longer than 72 h) and scheduled for hip internal fixation or hip arthroplasty by the same surgical team under spinal anesthesia. Postoperatively, all patients received intravenous patient-controlled analgesia with the same regimen (100 μg sufentanil and 8 mg ondansetron in 100 mL normal saline). A total of 110 adults were recruited in this study (**Figure 1**). Patients were excluded if they: (Daiello et al., 2019) had a past medical history of neurological disease such as delirium, schizophrenia, dementia, or stroke [with regard to dementia, it was screened if Mini Mental State Examination (MMSE) scores of $\leq$ 17 for illiterate patients, $\leq$ 20 for patients with 1–6 years of education, or $\leq$ 24 for patients with 7 or more years of education] (Li et al., 2016; Vutskits and Xie, 2016) were unable to read or write or cooperate; (Eckenhoff et al., 2020) had a history of drug or alcohol abuse. Eighty participants (15 POD vs. 65 Non-POD) completed the study. In the 15 POD patients, 5 POD patients' CSF were mixed with blood during the spinal anesthesia. In order to rule out the influence of blood on the results of CSF metabolomics and lipidomics to the greatest extent, we finally chose to analyze the 10 POD patients and the matched 30 Non-POD patients by age ($\pm$ 2 years) and MMSE score ($\pm$ 2 points), to make sure the two groups of patients are comparable. After admission, all patients were administered oxycodone/acetaminophen (5/325 mg, four times a day) to relieve pain.

Dietary Management

After admission, all patients were placed on a bland diet and began fasting at 22:00 on the night before surgery. Patients were started on a semiliquid diet 6 h after surgery and then subsequently advanced to a bland normal diet.

Anesthesia and Surgery

All participants underwent hip internal fixation or hip arthroplasty under spinal anesthesia. All surgeries were performed by the same surgical team to avoid potential

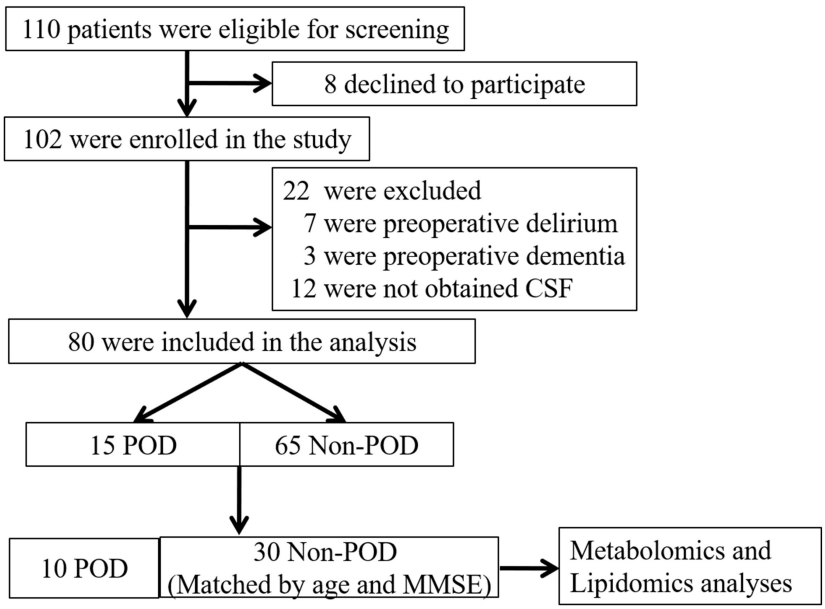

FIGURE 1 | Flow diagram showing the recruiting criterion. One hundred and ten patients were initially screened for the study, and 40 patients were finally included in the data analysis. CSF, cerebrospinal fluid; POD, postoperative delirium; MMSE, mini-mental state examination.

confounding factors caused by varying surgical skills or different surgical practices. Electrocardiography, pulse oximetry, and non-invasive blood pressure were all continuously monitored during anesthesia and were recorded at 3 min fixed intervals. Postoperatively, all patients received intravenous patient-controlled analgesia with the same regimen (100 μg sufentanil and 8 mg ondansetron in 100 mL normal saline).

Neuropsychological Testing

We interviewed all patients the day before surgery and performed MMSE. The Confusion Assessment Method (CAM) was used to identify patients who experienced preoperative delirium. CAM was performed twice daily on the first and second days after surgery (8:00 and 20:00). According to a previous study, POD was usually diagnosed in elderly hip fracture surgery patients during this period (Scholtens et al., 2016). The presence or absence of POD was defined according to the results of the Chinese version of CAM, which has good reliability and validity in the elderly Chinese population (Shi et al., 2014).

CSF Sample Preparation

After successful administration of spinal anesthesia, but prior to the administration of local anesthetic, CSF (2 mL) was collected in a polypropylene tube and placed on ice. Samples were immediately centrifuged at 3,000 × g for 10 min at 4°C to remove cells (Muller et al., 2014), and the supernatant was aliquoted and stored at −80°C until analysis. Metabolites and lipids were extracted from CSF samples using liquid–liquid extraction. Briefly, 500 μL of CSF were concentrated to 50 μL and mixed with a fourfold volume of ice-cold chloroform/methanol (2:1, v/v). After vortexing for 15 min at 4°C, the mixture was centrifuged at 13,000 × g for 15 min. The upper aqueous phase (hydrophilic metabolites) and lower organic phase (hydrophobic metabolites) were separately collected and evaporated at room temperature under vacuum. All evaporated samples were stored at −80°C until high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis.

High-Performance Liquid Chromatography

Metabolomics and lipidomics were performed on an Ultimate 3000 UHPLC system coupled with a Q-Exactive HF MS (Thermo Scientific, Waltham, MA). For the aqueous phase (metabolomics), an Xbridge amide column (100 × 2.1 mm i.d., 3.5 μm; Waters Corporation, Milford, MA) was employed for compound separation at 30°C. Samples were suspended in 100 μL of acetonitrile:water (1:1, v/v), and the injection volume was 10 μL. Mobile phase A consisted of 5 mM ammonium acetate in water with 5% acetonitrile, and mobile phase B was acetonitrile. The flow rate was 0.4 mL/min with the following linear gradient: 0 min, 95% B; 3 min, 90% B; 13 min, 50% B, 14 min, 50% B; 15 min, 95% B; and 17 min, 95% B.

As for lipids, chromatographic separation was performed on a reverse-phase X-select CSH C18 column (2.1 × 100 mm, 2.5 μm; Waters) at 40°C. Two solvents, both containing 10 mM ammonium acetate and 0.1% formic acid, were used for gradient elution: (A) ACN/water (3:2, v/v) and (B) IPA/ACN (9:1, v/v). The gradient program was: 0 min, 40% B; 2 min, 43% B; 12 min, 60% B; 12.1 min, 75% B; 18 min, 99% B; 19 min, 99% B; and 20 min, 40% B. The flow rate was set to 0.4 mL/min. Samples were suspended with 100 μL of chloroform:methanol (1:1, v/v) and diluted threefold with isopropanol:acetonitrile:H_2O (2:1:1, v/v/v). The injection volume was 10 μL.

Mass Spectrometry

Data-dependent acquisition (DDA) was performed using Q-Exactive HF MS (Thermo Scientific). For DDA-MS, acquisition was performed in the positive and negative switching ion mode under profile type. Each acquisition cycle consisted of 1 survey scan (MS1 scan) at 60,000 resolution from 60 to 900 m/z for hydrophilic metabolites and mass range m/z 300 to 1,200 for lipids, followed by 10 MS/MS scans in HCD mode at 30,000 resolution using stepped normalized collision energies of 15, 30, and 45. Dynamic exclusion was set to 8 s. The automatic gain control target was set to 5e6 (30 ms maximum injection time) and 2e5 (100 ms maximum injection time) for MS1 and MS/MS scans, respectively. Parameters of the ion source were: spray voltage of 3.3 kV for positive ion mode and 3.0 kV for negative ion mode; ion source sheath gas = 40; auxiliary gas = 10; capillary temperature = 320°C; probe heater temperature = 300°C; and S-lens RF level = 55. Samples were analyzed in random order. Quality control (QC) samples were prepared by pooling equal volumes of all study instances, and were analyzed between every five samples during the entire LC–MS analytical sequence.

DDA-MS Data Analysis

Raw data collected from DDA–MS were processed on MS–DIAL software (Tsugawa et al., 2015) according to the user guide. Briefly, raw MS data were converted from the vendor file format (.wiff) into the common file format of Reifycs Inc. (.abf) using the Reifycs ABF converter[1]. After conversion, MS–DIAL software was used for feature detection, spectra deconvolution, metabolite identification, and peak alignment between samples. MS/MS spectra-based metabolite identification was performed in MS–DIAL by searching the acquired MS/MS spectra against the MassBank database. It contains MS1 and MS/MS information of metabolites (3,928 records in positive ion mode and 4,963 records in negative ion mode). MS/MS spectra-based lipid identification was performed in MS–DIAL by searching the acquired MS/MS spectra against the internal *in silico* MS/MS spectra database. It includes MS1 and MS/MS information of common lipid species. Tolerances for MS1 and MS/MS searches were set to 0.01 and 0.05 Da, respectively. Other MS–DIAL parameters were set to default values.

Statistical Analysis

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were implemented to visualize the quality of metabolic profiling and metabolic differences between POD and Non-POD groups. Significantly altered metabolites with variable importance in projection (VIP) values >1 in the abovementioned PLS-DA model, as well as differing *p*-values determined by Student's *t*-test ($p < 0.05$), were selected in POD and Non-POD groups. PCA, PLS-DA, and pathway enrichment analysis for DDA data were performed with Metaboanalyst 4.0[2], an online tool for analyzing omics data. Furthermore, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify pathways associated with altered metabolites.

SPSS software (version 21.0; IBM Corporation, Armonk, NY) was used for data analysis. Data are expressed as mean ± SD, median and interquartile range (IQR), or number (%). The Kolmogorov–Smirnov method was used to test the normality of all variables. Categorical variables were analyzed using a χ^2-test, while continuous variables were analyzed using an independent-samples *t*-test. The Mann–Whitney *U*-test was used to analyze non-normal variables. Statistical significance was set at $p < 0.05$. Given the success of PLS-DA models in classifying POD and Non-POD groups, the top 20 differentially expressed lipids according to VIP were identified. Afterward, receiver operating characteristic (ROC) curves for dysregulated molecules were calculated in order to evaluate their potential use as candidate

[1]http://www.reifycs.com/AbfConverter/index.html

[2]https://www.metaboanalyst.ca

TABLE 1 | Subject characteristics.

	POD group (*n* = 10)	Non-POD group (*n* = 30)	Statistical test	*P*-value
Age (years), mean ± *SD*	82.2 ± 7.6	81.7 ± 7.2	*t* = 0.188	0.852
MMSE score, mean ± *SD*	25.2 ± 3.9	25.4 ± 3.6	*t* = -0.174	0.863
Male, *n* (%)	5 (50.0)	7 (23.3)	χ^2 = 1.429	0.232
Height (cm), mean ± *SD*	165.0 ± 7.9	163.2 ± 9.4	*t* = 0.516	0.609
Weight (kg), mean ± *SD*	66.1 ± 14.2	63.1 ± 10.5	*t* = 0.647	0.522
BMI (kg/m^2), mean ± *SD*	24.6 ± 5.4	23.3 ± 3.0	*t* = 0.846	0.404
ASA class, *n* (%)			χ^2 = 0.342	0.559
II	8 (80.0)	19 (63.3)		
III	2 (20.0)	11 (36.7)		
Education (years), median (IRQ)	13.5 (7.8)	9.0 (9.8)	*z* = -0.793	0.428
Length of anesthesia (min), mean ± *SD*	91.9 ± 12.5	93.8 ± 30.7	*t* = -0.171	0.865
Length of surgery (min), mean ± *SD*	63.8 ± 11.6	75.8 ± 33.4	*t* = -0.994	0.328
Charlson comorbidity score, mean ± *SD*	5.9 ± 1.0	6.3 ± 1.7	*t* = -0.671	0.506
Preoperative VAS score, mean ± *SD*	3.6 ± 1.0	3.2 ± 0.9	*t* = 0.786	0.441

POD, postoperative delirium; MMSE, Mini-Mental State Examination; ASA, American Society of Anesthesiologists; IQR, interquartile range; VAS, Visual Analogue Scale; cm, centimeter; kg, kilogram; min, minute; SD, standard deviation.

biomarkers for POD; the area under the curve (AUC) provides a global summary statistic of test accuracy.

RESULTS

Participant Characteristics

In this study, 10 CSF samples were collected from patients with CAM-confirmed POD, while 30 matched CSF samples were collected from patients without POD. There were no differences in age, MMSE score, gender, height, weight, body mass index, American Society of Anesthesiologists (ASA) physical class, education years, length of anesthesia and surgery, Charlson comorbidity score, or preoperative visual analog scale between POD and Non-POD groups (**Table 1**).

Untargeted Metabolic Profile Before Surgery

After processing the raw MS data, PCA analysis was used to create an overview of metabolomic expression profiles of all samples in positive and negative ion modes. Pooled QC samples were

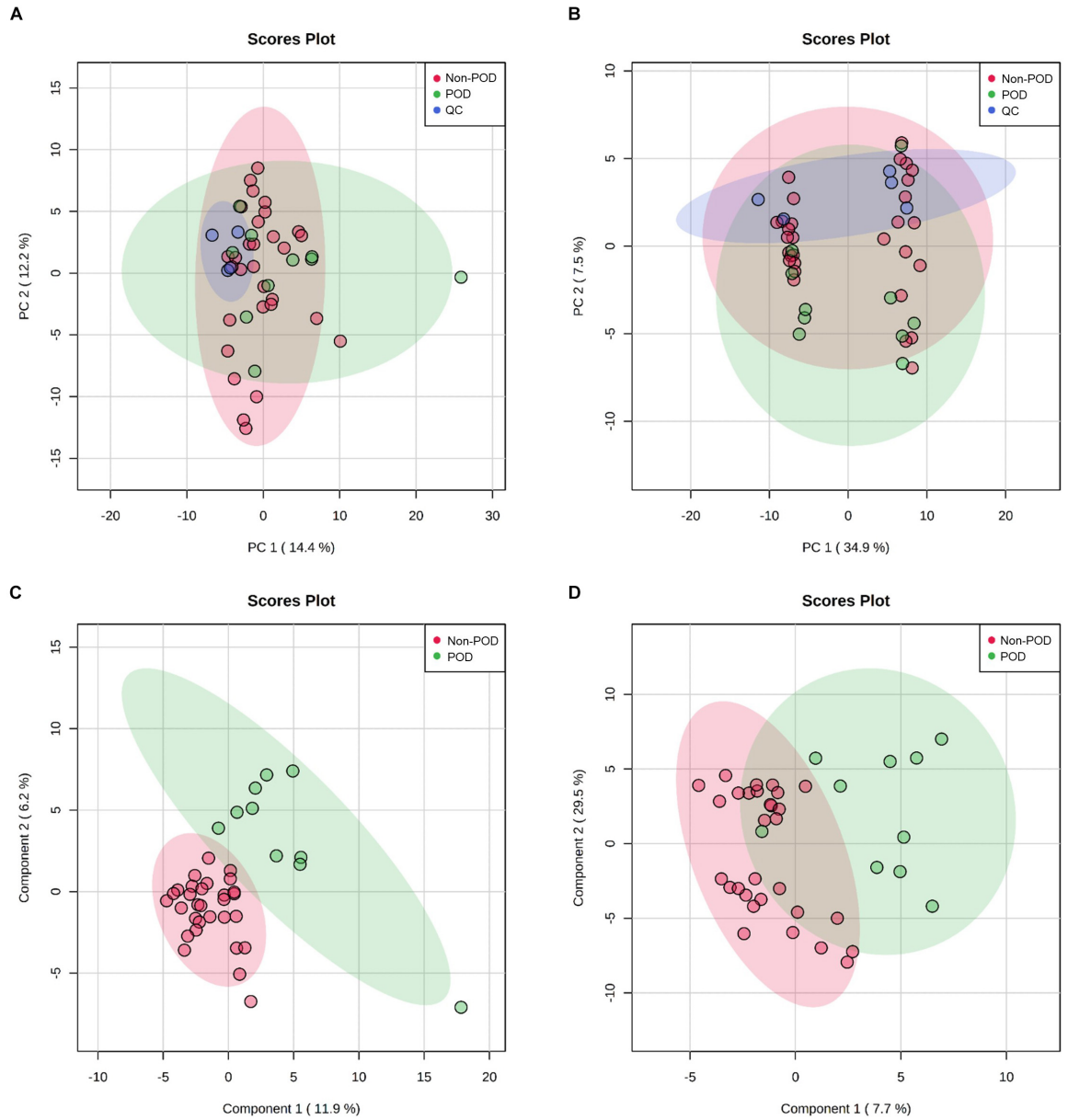

FIGURE 2 | Untargeted metabolic profiling of CSF samples in POD patients and Non-POD patients. PCA (**A:** positive-ion mode; **B:** negative-ion mode) and PLS-DA (**C:** positive-ion mode; **D:** negative-ion mode) analyses of the DDA-based metabolomics data. The indicated groups are presented by different colors (green: POD; red: Non-POD; blue: QC).

clustered well in the PCA score plots. In addition, POD and Non-POD groups clustered successfully in the PLS-DA model (**Figure 2**). After peak alignment and the removal of missing values, 202 features in positive ion mode and 156 in negative ion mode were reliably identified by MS/MS spectra comparison. Of these features, further statistical analysis revealed a total of 18 dysregulated metabolites between POD and Non-POD groups (VIP $>$ 1 and *t*-test $p < 0.05$, **Table 2**).

Untargeted Lipidomic Profile Before Surgery

As with the data processing procedures for metabolomic features, PCA and PLS-DA were used to generate an overview of the expression patterns of lipids in all samples (**Figure 3**). Similar to the observed results of metabolomic profiles, lipid QC samples were closely gathered in the PCA score plots in both positive and negative ion modes. In total, 385 features in positive ion mode and 348 in negative ion mode were reliably identified by MS/MS spectra comparison. Statistical analysis revealed 33 lipids (**Supplementary Table S1**) were dysregulated between POD and Non-POD groups (VIP $>$ 1 and *t*-test *p*-value < 0.05). The top 20 dysregulated lipids in POD patients according to VIP are shown in **Table 3**.

Bioinformatic Analysis Revealed Perturbed Metabolic Pathways

The 18 dysregulated metabolites and 20 dysregulated lipids were subjected to KEGG pathway enrichment analyses. Further bioinformatic analyses were employed to reveal perturbed pathways and provide clues for the underlying pathological mechanism. According to pathway impact and a *p*-value of < 0.05, the top six metabolic pathways significantly perturbed in POD patients (**Figure 4**) were D-glutamine and D-glutamate metabolism (two hits: glutamate and glutamine); glycerophospholipid metabolism [nine metabolites in four hits: phosphatidylethanolamine (PE) (40:6), PE (38:7e), PE (40:7e), phosphatidylcholine (PC) (34:3), PC (40:6), PC (32:2), PC (33:2), sn-glycero-3-phosphocholine, and rac-glycerol 3-phosphoate]; alanine, aspartate, and glutamate metabolism (three hits: glutamate, glutamine, and D-Glucosamine-6-phosphate); sphingolipid metabolism [two hits: sphingomyelin (SM) (d34:1) and SM (d42:2)]; histidine metabolism (two hits: histamine and glutamate); and arginine biosynthesis (two hits: glutamate and glutamine).

Metabolites Associated With Potential Biomarkers for POD

Further ROC analysis revealed four metabolites and eight lipids with an AUC greater than 0.8 (**Table 4**). In particular, PE (40:7e), which had the largest AUC value of 0.92, could be considered a potential biomarker for POD (**Figure 5**).

DISCUSSION

Hypotheses about POD development include "neuroinflammatory," "neuronal aging," "oxidative stress," "neurotransmitter deficiency," and "network disconnectivity" (Maldonado, 2013). Previous studies have found that aging, preoperative cognitive impairment, and dementia are the most important factors associated with POD. Our metabolomics results suggest that neuroinflammation, oxidative stress, energy metabolism disorders, and neurotransmitter imbalances caused by hypoxia and mitochondrial dysfunction at baseline may contribute to the risk of POD. Our current findings are not only consistent with previous pathophysiological models of delirium, but also extend these findings.

TABLE 2 | Differentiating metabolites between POD and Non-POD groups identified from the metabolomic data.

Pathway	Metabolites	VIP	*P*-value	FC (P/N)	Trend
Amino sugar metabolism	N-Acetylmannosamine	2.67	1.52E-03	0.39	Down
Amino acid metabolism	L-Saccharopine	2.60	1.88E-03	0.57	Down
Fatty acid metabolism	9-Trans-Palmitelaidic acid	2.53	2.64E-03	2.58	Up
Fatty acid metabolism	Citramalate	2.51	2.82E-03	0.66	Down
Glycolysis	D-Glucose 6-phosphate	2.23	8.85E-03	2.94	Up
Amino acid metabolism	Creatine	2.21	9.51E-03	0.75	Down
Histidine metabolism	Histamine	2.21	1.00E-02	0.47	Down
Amino acid metabolism	Methionine	2.21	1.01E-02	0.57	Down
Fatty acid metabolism	Trans-Vaccenic acid	2.2	1.02E-02	0.88	Down
Amino sugar metabolism	N-Acetylgalactosamine	2.14	1.28E-02	0.26	Down
Amino acid metabolism	Glutamate	2.14	1.32E-02	4.21	Up
Purine metabolism	Hypoxanthine	2.11	1.41E-02	0.67	Down
Amino acid metabolism	N-Methylproline	1.98	2.28E-02	0.47	Down
Amino acid metabolism	Glutamine	1.96	2.33E-02	0.64	Down
Lipid metabolism	rac-Glycerol 3-phosphoate	1.91	2.70E-02	0.60	Down
Lipid metabolism	sn-Glycero-3-phosphocholine	1.86	3.25E-02	1.54	Up
Amino sugar metabolism	D-Glucosamine-6-phosphate	1.79	4.02E-02	0.63	Down
Amino acid metabolism	Dopamine	1.71	4.94E-02	13.15	Up

VIP, variable importance in the projection; FC, fold change.

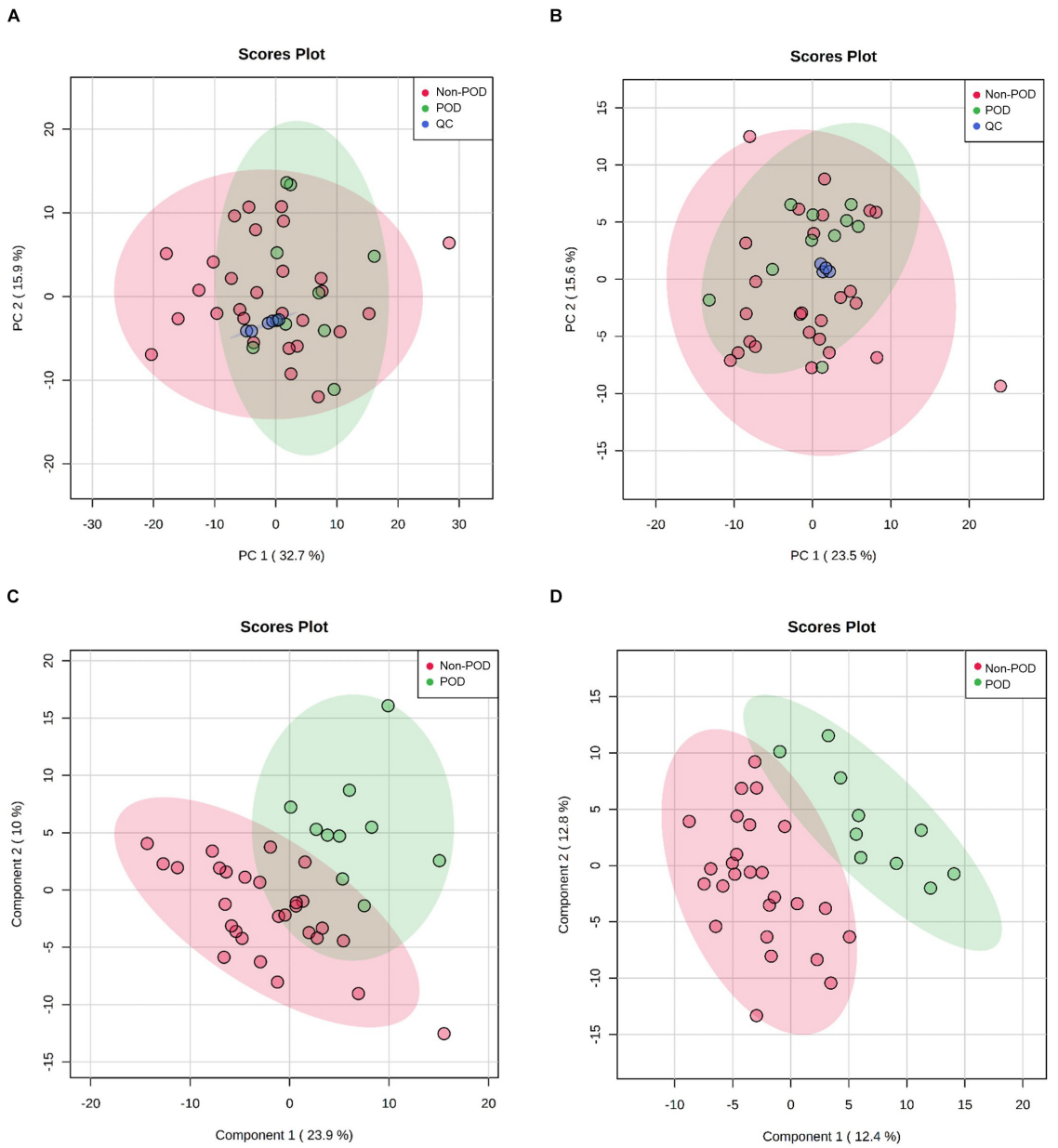

FIGURE 3 | Untargeted lipidomics profiling of CSF samples in POD patients and Non-POD patients. PCA (**A:** positive-ion mode; **B:** negative-ion mode) and PLS-DA (**C:** positive-ion mode; **D:** negative-ion mode) analyses of the DDA-based lipidomics data. The indicated groups are presented by different colors (green: POD; red: Non-POD; blue: QC).

Hypoxia impairs normal function of the blood–brain barrier and promotes neurodegeneration. The storage of adenosine triphosphate (ATP) in brain tissue is very limited. If an insufficient supply of ATP is produced as a result of hypoxia or an insufficient oxygen supply, glycolysis can be promoted to compensate for this deficiency. The first step of glycolysis is the formation of glucose-6-phosphate, which is catalyzed by hexokinase. In this study, we found that the key intermediate product of glycolysis (the main pathway of carbohydrate metabolism), glucose-6-phosphate, was significantly increased in the CSF of POD patients compared with Non-POD patients (FC POD/Non-POD = 2.94, p = 8.85E-03), indicating that the brain tissue of POD patients might exist in a hypoxic environment. In addition, our results suggest that hypoxanthine, which is involved in the purine metabolic pathway, was decreased in the CSF of POD patients compared with Non-POD patients (FC POD/Non-POD = 0.67, p = 0.01). Moreover, disturbances in nucleic acid metabolism were observed. Levels of inosine in preoperative CSF also exhibited a decreasing trend in POD patients (FC POD/Non-POD = 0.82, p = 0.06), partially reflecting the enhanced oxidative damage to cell nuclei and mitochondrial DNA.

TABLE 3 | The top 20 differentiating lipids between POD and Non-POD groups identified from the lipidomic data.

Pathway	Lipids	VIP	*P*-value	FC (P/N)	Trend
Sphingolipid metabolism	Cer-NS (d40:2); Cer-NS (d18:1/22:1)	2.96	4.41E-05	2.51	Up
Glycerophospholipid metabolism	PE (40:6); PE (18:0–22:6)	2.86	8.00E-04	0.59	Down
Glycerophospholipid metabolism	PE (38:7e); PE (16:1e/22:6)	2.76	1.35E-03	0.66	Down
Glycerophospholipid metabolism	PE (40:7e); PE (18:1e/22:6)	2.70	1.74E-03	0.43	Down
Glycerolipid metabolism	DAG (44:5e); DAG (22:3e/22:2)	2.61	4.52E-04	0.47	Down
Glycerolipid metabolism	LDGCC (34:1)	2.49	9.05E-04	0.6	Down
Sphingolipid metabolism	Cer-NS (d50:1); Cer-NS (d22:1/28:0)	2.39	1.61E-03	2.18	Up
Sphingolipid metabolism	Cer-NS (d52:1); Cer-NS (d22:1/30:0)	2.27	2.89E-03	3.00	Up
Sphingolipid metabolism	Cer-NS (d42:4); Cer-NS (d22:3/20:1)	2.26	3.03E-03	1.79	Up
Glycerophospholipid metabolism	PC (34:3)	2.18	4.47E-03	75.52	Up
Glycerophospholipid metabolism	PC (40:6); PC (18:0–22:6)	2.12	1.69E-02	0.51	Down
Glycerophospholipid metabolism	PC (33:1); PC (16:0–17:1)	2.06	2.04E-02	0.74	Down
Glycerophospholipid metabolism	PC (32:2)	2.02	8.79E-03	1.60	Up
Sphingolipid metabolism	Sphinganine (25:0)	2.02	9.11E-03	0.42	Down
Sphingolipid metabolism	SM (d34:1); SM (d18:1/16:0)	1.96	1.17E-02	4.78	Up
Sphingolipid metabolism	SM (d44:2); SM (d21:2/23:0)	1.92	1.34E-02	1.59	Up
Sphingolipid metabolism	SM (d34:2); SM (d14:2/20:0)	1.84	1.84E-02	1.26	Up
Glycerophospholipid metabolism	PC (37:3)	1.82	1.99E-02	15.33	Up
Glycerophospholipid metabolism	PC (33:2)	1.80	2.14E-02	1.65	Up
Sphingolipid metabolism	SM (d42:2); SM (d18:1/24:1)	1.79	2.18E-02	1.27	Up

VIP, variable importance in the projection; FC, fold change; Cer-NS, ceramide non-hydroxyfatty acid-sphingosine; PE, phosphatidylethanolamine; DAG, diacylglycerol; LDGCC, lysodiacylglyceryl-3-O-carboxyhydroxymethylcholine; PC, phosphatidylcholine; SM, sphingomyelin.

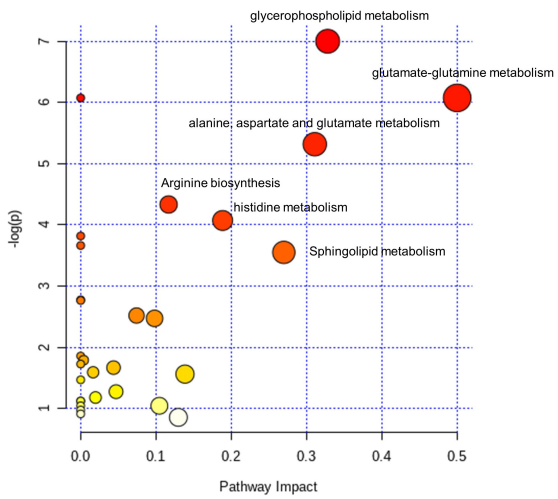

FIGURE 4 | Pathway analysis of the dysregulated metabolites and lipids in the CSF of POD patients. Fifteen selected metabolites and lipids were involved in the D-Glutamine and D-glutamate metabolism (2 hit), Glycerophospholipid metabolism (4 hits), Alanine, aspartate and glutamate metabolism (3 hits), Sphingolipid metabolism (2 hits), Histidine metabolism (2 hits) and Arginine biosynthesis (2 hits). The color gradient indicates the significance of the pathway ranked by *p*-value (y-axis; yellow: higher *p*-values, red: lower *p*-values), and circle size indicates the pathway impact score (x-axis; the larger circle, the higher impact score). Significantly affected pathways with low *p*-values and high pathway impact scores are labeled.

When ATP is exhausted, creatine can be rapidly metabolized to provide energy for nerve cells. Because insufficient ATP was produced by glycolysis in the hypoxic environment of POD patients, creatine was significantly consumed (FC POD/Non-POD = 0.75, p = 9.51E-03) to supplement ATP production. Deficiency of energy metabolism could induce cerebral dysfunction, as well as the associated cognitive and behavioral symptoms of delirium. Decreased oxygenation causes a failure in oxidative metabolism, which may be one cause of the problems observed in delirium, namely oxidative stress, which causes a failure of the ATPase pump system. When the pump fails, ionic gradients cannot be maintained, leading to significant influxes of sodium (Na^+) followed by calcium (Ca^{2+}), whereas potassium (K^+) moves out of the cell (Siesjo, 1984; Kirsch et al., 1989). The influx of Ca^{2+} during hypoxic conditions is associated with a dramatic release of several neurotransmitters, particularly dopamine (DA) and glutamate (Glu). Neurotransmitter disturbances may be primary contributors to POD development. It is commonly accepted that neurotransmitter imbalances in the brain are the final common pathway in the occurrence of delirium. Thus, the pathogenesis of POD might be associated with alterations in neurotransmitter synthesis, function, and/or availability that mediate complex behavioral and cognitive changes. The most commonly described neurotransmitter changes associated with delirium include excess DA and/or Glu release.

DA, an endogenous central neurotransmitter, is the immediate precursor of norepinephrine in the catecholamine synthesis pathway. Excess DA is among the most commonly described neurotransmitter imbalances in the pathogenesis of delirium (Maldonado, 2013). Increased dopaminergic transmission is usually accompanied by reduced central cholinergic transmission, which is postulated to be associated with delirium. Yilmaz et al. (2016) studied 137 patients undergoing coronary

TABLE 4 | AUCs for metabolites and lipids.

Metabolites or lipids	AUC	95% CI	*P* Value	SE
PE (40:7e); PE (18:1e/22:6)	0.92	0.84–1.00	9.20E-05	0.04
Cer-NS (d40:2); Cer-NS (d18:1/22:1)	0.90	0.80–1.00	2.05E-04	0.05
DAG (44:5e); DAG (22:3e/22:2)	0.88	0.77–0.99	4.42E-04	0.06
PE (38:7e); PE (16:1e/22:6)	0.84	0.71–0.98	1.46E-03	0.07
PE (40:6); PE (18:0–22:6)	0.84	0.70–0.98	1.64E-03	0.07
L-Saccharopine	0.84	0.67–1.00	1.46E-03	0.09
PC (40:6); PC (18:0–22:6)	0.83	0.69–0.96	2.56E-03	0.07
N-Acetylmannosamine	0.83	0.67–0.98	2.56E-03	0.08
Sphinganine (25:0)	0.82	0.69–0.96	2.85E-03	0.07
9-Trans-Palmitelaidic acid	0.81	0.67–0.95	4.36E-03	0.07
Cer-NS (d42:4); Cer-NS (d22:3/20:1)	0.80	0.65–0.96	4.84E-03	0.08
Citramalic acid	0.80	0.63–0.98	4.84E-03	0.09

AUC, area under the curve; 95% CI, 95% confidence interval; SE, standard error; PE, phosphatidylethanolamine; Cer-NS, ceramide non-hydroxyfatty acid-sphingosine; DAG, diacylglycerol; PC, phosphatidylcholine.

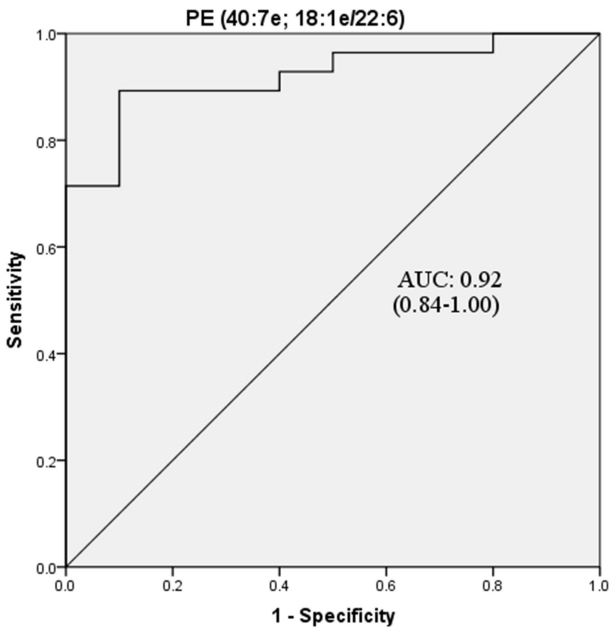

FIGURE 5 | ROC curve analysis of potential CSF biomarker for differentiating the POD group from the Non-POD group. PE, phosphatidylethanolamine. The area under the curve for the prediction of POD via PE (40:7e) was 0.92 (95% CI = 0.84–1.00).

artery bypass surgery and found that intraoperative DA was an independent risk factor for POD. Relative risk was 2.37 (95% CI: 0.18–31.28; p = 0.51) for total doses over 10 mg/kg and 3.55 (95% CI: 1.16–10.89; p = 0.02) for total doses over 30 mg/kg, indicating that high-dose DA was more likely to cause POD. In this study, we found that the CSF DA level was significantly increased in POD patients compared with Non-POD patients (FC POD/Non-POD = 13.15, p = 0.04), consistent with previous studies. Under impaired oxidative conditions, significant amounts of DA are released and there is a failure of adequate DA reuptake. An influx of Ca^{2+} stimulates the activity of tyrosine hydroxylase, which converts tyrosine to 3,4-dihydroxyphenylalanine to increase DA production, and further uncouples oxidative phosphorylation in brain mitochondria (Kirsch et al., 1989). Kesby et al. (2018) found that higher dopamine metabolites were associated with psychotic features. Elevation in DA availability may lead to some of the observed neurobehavioral alterations via the direct excitatory activity effects of DA, inducing apoptosis by mechanisms independent of oxidative stress (Bagnoli et al., 2020) and potentiation of the excitotoxic effects of Glu (Belov Kirdajova et al., 2020).

Widely recognized as the primary excitatory neurotransmitter in the brain, Glu plays crucial roles in cognitive function and the development of neurodegenerative disorders. Guo et al. (2017) conducted a clinical study of preoperative serum metabolites associated with POD in elderly hip fracture patients and found that preoperative serum glutamine (Gln) was significantly decreased in POD patients (FC POD/Non-POD = 0.90, p = 0.02). We observed similar results in preoperative CSF. The Glu level in CSF samples of POD patients was markedly increased (FC POD/Non-POD = 4.21, p = 0.01), while the Gln level was significantly decreased (FC POD/Non-POD = 0.64, p = 0.02), indicating that Glu–Gln cycle homeostasis might be disturbed, thus giving rise to cognitive impairment in elderly patients. Normally, Glu is released into the synapse, removed by astrocytes, and subsequently converted into Gln, ending its action. However, under oxidative conditions, Glu accumulates in the extracellular space because its reuptake and metabolism in glial cells is impeded by ATPase pump failure (Herrera-Marschitz et al., 2018).

Lipids have diverse biological functions, such as cellular architecture, energy storage, and cell signaling (Shevchenko and Simons, 2010) and brain lipid homeostasis plays an important role in AD and other neurodegenerative disorders. The most abundant lipid species identified in the central nervous system are cholesterol, ceramide (Cer), sphingomyelin (SM), phosphatidylcholine (PC), glucosyl ceramides, and sulfatides (Zarrouk et al., 2018). It is well-established that Cer is involved in oxidative stress, inflammation, and/or cell death, which contribute to the development of AD. Apoptosis is stimulated

by Cer though the inhibition of mitochondrial electron transport and cytochrome release (Faezi et al., 2015). High levels of Cer in the brain can promote inflammatory pathways and exert negative effects on neurons during the aging process (Cutler et al., 2004). A previous study found that cytokines increased hepatic SM synthesis to increase plasma SM levels (Memon et al., 1998); moreover, elevated levels of SM have been reported in AD brains (El Gaamouch et al., 2016). Mielke et al. (2010) conducted a longitudinal population-based study involving 100 women who were followed up in six visits over 9 years. They found that serum Cer and SM varied according to the timing of memory impairment onset and may be good pre-clinical predictors (or biomarkers) of memory impairment – a deficit observed early in AD pathogenesis. Mapstone et al. (2014) performed a targeted metabolomic and lipidomic analysis and found that PC (36:6), PC (38:0), PC (38:6), PC (38:0), PC (40:1), PC (40:2), and PC (40:6) were depleted in the plasma of the Converter group, but not in the Non-Converter group. As the suppression of PC composition is itself sufficient to result in apoptosis, lacking some types of PC can be a critical factor leading to neuronal damage (Adibhatla and Hatcher, 2002). The relationship between inflammatory markers and the lipidome has been investigated. Wallace et al. performed a lipidomic analysis to study the relationship between the lipidome and inflammatory markers, and found strong negative correlations between inflammatory markers (C-reactive protein and tumor necrosis factor α) and lipids (PE and PC classes), whereas interleukin 8 had positive correlations with lipids of Cer and SM classes (Wallace et al., 2014). In our study, we found similar results indicating that preoperative CSF levels of PE and PC classes, including PE (40:6), PE (38:7e), PE (40:7e), PC (40:6), and PC (33:1), were significantly decreased in the POD group compared with the Non-POD group. However, in POD patients, preoperative CSF levels of Cer-NS and SM classes were significantly increased ($P < 0.05$). These results indicate that levels of inflammatory markers may be increased in the preoperative CSF of POD patients, and neuroinflammation plays an important part in the progression of POD.

In conclusion, we revealed the presence of alterations in multiple metabolic pathways in the POD group before surgery, including neuroinflammation, oxidative stress, and energy metabolism, which involve interactions between hypoxia and mitochondrial dysfunction, as well as neurotransmitter imbalances. These metabolic abnormalities potentially increase the fragility of the brain and contribute to POD.

LIMITATIONS

Our study has some limitations. First, power calculations were not performed. Instead, we aimed to recruit the maximum number of patients available. We only recruited 10 POD patients and 30 Non-POD patients in our metabolomic and lipidomic analysis. Thus, further validation analysis needs to be performed in larger cohorts in future studies. Second, this work could be improved by performing the multiple reaction monitoring- mass spectrometry (MRM-MS) validation on some of the metabolites and lipids between POD and Non-POD patients. Considering the ethical issue of limited CSF, we regret to say that there were not enough CSF samples, especially POD patients' samples, for MRM-MS validation after metabolomic and lipidomic analyses. We can only do this in the future after we have obtained enough CSF. Although we did not perform the MRM-MS validation, quality control samples were prepared by pooling equal volumes of all study instances, and were analyzed between every five samples during the entire LC–MS analytical sequence. The concentration data were normalized to sample median and take the logarithm. The methodology at each step was as rigorous as we could. We hope that researchers can pay attention to it and provide reference for more rigorous clinical design in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Beijing Jishuitan Hospital Medical Science Research Ethics Committee (JLKS201901-04). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH, ZL, YZ, ML, and XG designed the study. YH, WZ, YS, TL, GW, and YY collected samples and performed clinical-related analyses. JL and LZ performed metabolomic and lipidomic experiments. YH, XW, NY, YL, XM, and DH reviewed statistical analyses. YH and JL wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.570210/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cognitive Reserve, Leisure Activity, and Neuropsychological Profile in the Early Stage of Cognitive Decline

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In older adults with normal cognition, cognitive reserve (CR) is known to be associated with the neuropsychological profile. We investigated the association between comprehensive CR and detailed neuropsychological profile in the early stage of cognitive decline. Fifty-five participants with mild cognitive impairment or subjective cognitive decline completed the cognitive reserve index questionnaire (CRIq) that yielded total, education, working activity, and leisure time scores (CRI-Total, CRI-Education, CRI-Working activity, and CRI-Leisure time, respectively). Mini-mental state examination (MMSE) and detailed neuropsychological evaluation were performed. Psychiatric symptom scales were applied to measure depression, apathy, positive or negative affect, and quality of life. Correlation and linear regression analyses of the variables were performed. The effect of CR-Education, CRI-Working activity, and CRI-Leisure time on the composite cognitive score was determined using a multivariable regression model. We observed that for CRI-Total (B = 3.00, p = 0.005), CRI-Education (B = 3.39, p = 0.002), and CRI-Leisure time (B = 2.56, p = 0.015), CR correlated with MMSE scores, while only CRI-Leisure time associated with the naming ability (B = 2.20, p = 0.033) in the detailed neuropsychological test results of the participants. Multivariable regression model also indicated that among CRI subscores, CRI-Leisure time directly affects the composite cognitive score (β = 0.32, p = 0.011). We found that in the early stage of cognitive decline in older adults, comprehensive CR was associated with global cognition, and only leisure activity was identified to be associated with the detailed neuropsychological profile including naming ability. These results may imply the positive effect of leisure activity on cognitive function in the early stages of cognitive decline.

Keywords: cognitive reserve, neuropsychological tests, leisure activity, cognition, mild cognitive impairment

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Abbreviations: AD, AlzheimerŠs disease; AES, apathy evaluation scale; CDR-SOB, clinical dementia rating-sum of boxes; COWAT, controlled oral word association test; CR, cognitive reserve; CRI, cognitive reserve index; CRIq, cognitive reserve index questionnaire; GDepS, geriatric depression scale; GDS, global deterioration scale; IQ, intelligence quotient; K-BNT, Korean version of the Boston naming; MMSE, mini-mental state examination; MCI, mild cognitive impairment; PANAS, positive and negative affect schedule; RCFT, Rey-Osterrieth complex figure test; RMSEA, root-mean-square error of approximation; SCD, subjective cognitive decline; SCLT, Seoul verbal learning test; TMT Ű A, trail making test-A.

INTRODUCTION

Aging and cognitive decline are major medical and social issues. The number of older adults will continue to grow worldwide, increasing the burden of aging (Pison, 2019). Approximately 10% of elderly people experience dementia, a common clinical syndrome often caused by underlying neurodegenerative diseases (Alzheimer's Association, 2016). The burden of dementia increases because dementia is a syndrome deteriorating cognition, behavioral, and psychological symptoms, and activities of daily living (American Psychiatric Association, 2013). Alzheimer's disease (AD), is the most prevalent form of dementia in elderly people, and to date, many efforts to develop effective treatments for AD have failed. Thus, preventing dementia is gradually gaining more support (Cummings et al., 2018).

It is known that the cognitive reserve (CR) can enhance the cognitive function against normal and pathologic aging (Stern, 2002; Scarmeas and Stern, 2004; Soldan et al., 2020). CR is a modifiable factor that can be changed or improved, and this is the basic theory behind cognitively, mentally, and physically stimulating activities to delay cognitive decline and dementia (Reed et al., 2010; Clare et al., 2017). CR has been measured by factors such as premorbid intelligence quotient (IQ), years of education, complexity of occupation, and composites of hobbies and leisure activities (Jones et al., 2011). It is well known that older adults with a higher level of education show better global and detailed neuropsychological function (Thow et al., 2017; Groot et al., 2018; Gu et al., 2018; Lavrencic et al., 2018; Zhang et al., 2019; Zarantonello et al., 2020) than those with lower education levels. Healthy lifestyle, including cognitive, social, and physical activities, has also been positively correlated with global cognition (Clare et al., 2017).

In terms of measurements, CR proxies such as education (MacPherson et al., 2017; Rodriguez et al., 2019; Zhang et al., 2019), IQ (Ghaffar et al., 2012; MacPherson et al., 2017), and occupational attainment (Ghaffar et al., 2012; Boots et al., 2015) have been used to represent a component of CR (Jones et al., 2011) rather than a comprehensive lifetime cognitive stimulating activity. In addition, previous studies have determined the effect of CR on cognitive function, as measured by global cognitive scales like the mini-mental state examination (MMSE) (Zhang et al., 2019) and the Montreal cognitive assessment (Park et al., 2019), and few have identified the relationship with comprehensive cognitive domains. Thus, an extended and thorough assessment of CR and a comprehensive neuropsychological evaluation may help to find their relationship. Additionally, most studies investigating CR and cognition were conducted in cognitively normal adults. Since educational and occupational attainment were found to affect the progression to dementia in patients with mild cognitive impairment (MCI) (Allegri et al., 2010; Myung et al., 2017), CR may play a protective role at the preclinical or prodromal stage of dementia that manifests with subtle decline of cognitive and psychiatric functions (Lyketsos et al., 2002; Robert et al., 2006).

We hypothesized that a higher comprehensive CR may correlate with a better detailed neuropsychological profile and psychiatric status in the early stage of cognitive decline. Hence, we assessed the association between comprehensive CR and a detailed neuropsychological profile and psychiatric symptoms, including depression, affect, and apathy.

MATERIALS AND METHODS

Participants

From May 2019 to December 2019, individuals with subjective cognitive decline (SCD) or MCI were prospectively recruited from the Memory Clinic at Gil Medical Center, Gachon University. All participants complained of subjective cognitive impairment and were diagnosed with either SCD based on the clinical evaluation [clinical dementia rating-sum of boxes (CDR-SOB) $\leq$ 0.5] and neuropsychological test results (all cognitive domain z-score $>$-1.5 standard deviation), as previously described (Jessen et al., 2014; Molinuevo et al., 2017), or with MCI according to Petersen's criteria (Petersen, 2004). The final diagnoses in the participants were confirmed by a board-certified psychiatrist (JM Kang). Patients with any of the following conditions were excluded: (1) Korean version of the MMSE score $<$ 20; (2) CDR-SOB score $>$4.0; (3) impaired activities of daily living; (4) major psychiatric disorders; (5) history of diagnosis with any kind of dementia or cerebrovascular diseases; (6) severe medical or surgical comorbidities that may affect cognition such as cancer, chronic kidney disease, chronic obstructive pulmonary diseases, and the acute phase after any major surgery; and (7) history of neurodegenerative disorders, including Parkinson's, Huntington's, Pick's, and Creutzfeldt-Jakob diseases. All participants provided written informed consent, and the Institutional Review Board of Gil Medical Center, Gachon University, approved the study (GAIRB2019-230).

Assessments

All participants were evaluated for CR, comprehensive neuropsychological and clinical functions, and psychiatric symptoms. To evaluate CR, we used the cognitive reserve index questionnaire (CRIq) that was developed by Nucci et al. (2012) and validated in Korean normal adults (Choi et al., 2016). This questionnaire consists of 20 questions yielding scores for each of the following domains: years of education both formal and non-formal (CRI-Education); working activity (CRI-Working activity), which classifies working activities into five levels depending on the cognitive load required for the job involved and the number of years spent in each occupation; leisure time (CRI-Leisure time), to assess the type and frequency of cognitive activities such as reading books, attending concerts, and caring for pets the participants spend their free time on; and a total score (CRI-Total), with an average of 100 and a standard deviation of 15 for each score. Scores obtained for each domain were then adjusted for age.

Neuropsychological function was evaluated in all participants. For evaluation of the functional daily activity, CDR (score range of 0–3), CDR-SOB (score range of 0–15), and global deterioration scale (GDS; score range of 0–7) were also

applied. The comprehensive neuropsychological tests consisted of subtests from the comprehensive neuropsychological test battery (Kang and Na, 2003). The digit span test and trail making test-A (TMT-A) were used to assess attention and the Seoul verbal learning test (SVLT) was used to assess verbal memory function (Kang and Na, 2003). The Rey-Osterrieth complex figure test (RCFT) copy test (Meyers and Meyers, 1995; Kang and Na, 2003) and the Korean version of the Boston naming test (K-BNT) (Kim and Na, 1999; Kang and Na, 2003) were used to assess visuospatial function and language ability, respectively. To evaluate the frontal executive functions, we used the TMT-B, controlled oral word association test (COWAT) animal, COWAT phonemic, and the Stroop test (color/word reading) (Kang and Na, 2003). Each neuropsychological test score was converted to a *z*-score based on its deviation from the overall score for the Korean elderly population with normal cognition of the same age and years of education. Additionally, a composite cognitive score and cognitive domain scores were calculated based on all the neuropsychological tests mentioned above that were validated for constructing a composite score in the Korean population (Jahng et al., 2015) by averaging standardized scores for each subtest (Bransby et al., 2019). All tests were evaluated by a board-certified neuropsychologist (SY Lee).

Psychiatric symptoms were also assessed. The geriatric depression scale (GDepS) was used to evaluate depressive symptoms. The GDepS is a validated, yes or no, 30-item questionnaire on mood, energy, anxiety, hopefulness, satisfaction, inattention, and insomnia, with higher scores indicating severe depression with a score range of 0–30 (Yesavage et al., 1982; Bae and Cho, 2004). The apathy evaluation scale (AES) was used to evaluate apathy. The AES is a validated, four-point Likert scale, 18-item questionnaire regarding apathy in the affective, behavioral, and cognitive aspects, with lower scores indicating severe apathy with a score range of 18–72 (Marin et al., 1991; Lee et al., 2013). The positive and negative affect schedule (PANAS) was used to evaluate affective symptoms. The PANAS is a validated, five-point Likert scale, 20-item questionnaire regarding alertness, enthusiasm, lethargy, and sadness (Watson et al., 1988; Lim et al., 2010). The PANAS yields scores for two domains, positive affect (PANAS-P) and negative affect (PANAS-N), with higher scores in PANAS-P indicating a higher positive affect with a score range of 10–50 and higher scores in PANAS-N indicating a higher negative affect with a score range of 10–50. The quality of life-Alzheimer's disease (QOL-AD), validated in people with dementia, was used to evaluate the QOL of the participants. The QOL-AD consists of 13 items regarding physical health, friends, living situation, and ability to do things for fun, with higher scores indicating a better QOL with a score range of 13–52 (Thorgrimsen et al., 2003; Shin, 2006).

Statistical Analyses

Demographic information and clinical data were analyzed with descriptive analysis. Correlations between CR and cognitive and psychiatric symptoms were analyzed using Pearson's correlation coefficients in data with normal distribution and Spearman's correlation coefficients in data with skewed distribution. Multiple comparisons were corrected by Benjamini–Hochberg false discovery rate method (Benjamini and Hochberg, 1995) in neuropsychological test results and psychiatric symptoms. Regression analyses were performed to determine the effect of CRIq scores on neuropsychological and psychiatric symptom test scores. To adjust covariates, sex and CDR-SOB or MMSE (for predicting CDR-SOB) were defined as independent variables as well as CRIq score, and each of the neuropsychological profiles or psychiatric symptoms were defined as a dependent variable. Since age and years of education were already adjusted as part of the neuropsychological test results and were included in the CRIq scores (Kang and Na, 2003; Choi et al., 2016), the dependent variables were only adjusted for sex. In the regression analyses, log transformation was performed for variables that did not distribute normally. The effect of each domain of CRIq on the composite cognitive score and subdomains of neuropsychological tests was determined using a path diagram of the multivariable regression model. A non-significant chi-square (χ^2), a root-mean-square error of approximation (RMSEA) under 0.05, and a Comparative Fit Index (CFI) value above 0.95 indicated a strong model fit for the multivariable regression model, while a CFI above 0.90 and an RMSEA value under 0.08 indicated an adequate fit (Hu and Bentler, 1999; Kline, 2015). All analyses were performed with SPSS for Windows (SPSS, version 23; Chicago, IL, United States) except for the multivariable regression model, which was performed using AMOS for Windows (IBM SPSS, version 20; Chicago, IL, United States). *P* values < 0.05 (two-way) were considered significant.

RESULTS

Demographic and Clinical Characteristics

Among the 58 participants who were enrolled in the study, two who failed to complete the assessments and one with a CDR-SOB score >4 were excluded from the analysis. Participants were then divided into two diagnostic groups: SCD ($n = 36$) and MCI ($n = 19$). **Table 1** shows the demographics and the global cognitive scale scores for the participants. Mean age was 74.06 ± 6.43 in SCD and 75.42 ± 5.98 in MCI groups ($t = -0.77$, $p = 0.446$). Individuals with SCD were more likely to be male ($\chi^2 = 4.52$, $p = 0.034$) with significantly higher MMSE ($Z = -2.53$, $p = 0.011$) and lower CDR-SOB ($\chi^2 = 18.41$, $p = 0.018$) scores.

CR, Cognitive Function, and Psychiatric Symptoms

Table 2 shows the results of the CRIq, comprehensive neuropsychological tests, and psychiatric symptom scales. There were no differences of CRIq scores between SCD and MCI groups except for CRI-Working activity that was higher in the SCD group ($Z = -2.10$, $p = 0.036$). The comprehensive neuropsychological test results were generally higher in the SCD group, while psychiatric symptoms were comparable in both groups. Cognitive domain scores in both groups are presented in **Supplementary Table S1** in **Supplementary Material**.

TABLE 1 | Demographics and clinical characteristics of the participants.

		SCD (n = 36)		MCI (n = 19)		t, Z, or χ^2, p
		Mean ± SD or number	Min, max or percent	Mean ± SD or number	Min, max or percent	
Age, years†		74.06 ± 6.43	62, 90	75.42 ± 5.98	64, 82	-0.77, 0.446
Sex (female; *n*,%)		20	55.6%	16	84.2%	4.52, 0.034*
Education, years		8.31 ± 4.49	0.5, 23	8.00 ± 4.03	0, 12	-0.41, 0.680
MMSE (SR: 0–30)		27.03 ± 2.20	20, 30	24.84 ± 3.18	20, 28	-2.53, 0.011*
GDS	1	7	19.4%	4	21.1%	13.40, 0.004**
(*n*,%; SR: 1–7)	2	21	58.3%	3	15.8%	
	3	8	22.2%	9	47.4%	
	4	0	0.0%	3	15.8%	
CDR	0	7	19.4%	3	15.8%	0.03, 0.987
(*n*,%; SR: 0–3)	0.5	29	80.6%	14	73.7%	
	1	0	0.0%	2	10.5%	
CDR-SOB	0	7	19.4%	3	15.8%	18.41, 0.018*
(*n*,%; SR: 0–15)	0.5	19	52.8%	3	15.8%	
	1	7	19.4%	5	26.3%	
	1.5	1	2.8%	2	10.5%	
	2	1	0.0%	0	0.0%	
	2.5	0	0.0%	3	15.8%	
	3	1	2.8%	0	0.0%	
	3.5	0	0.0%	2	10.5%	
	4	0	0.0%	1	5.3%	

*$p < 0.05$, **$p < 0.01$.† Normal distribution. Data are mean ± SD (minimum–maximum) or number (percent,%). Independent t test for continuous variables with normal distribution, Mann–Whitney U test for continuous variables without normal distribution, and chi-square test for categorical variables were used. SCD, subjective cognitive decline; MCI, mild cognitive impairment; SD, standard deviation; min, minimum; max, maximum; MMSE, mini-mental state examination; GDS, global deterioration scale; CDR, clinical dementia rating; CDR-SOB, clinical dementia rating-sum of boxes; SR, score range.*

Correlation Between CR and Cognitive Function or Psychiatric Symptoms

Table 3 shows the correlation between CRIq scores and neuropsychological test results and psychiatric symptoms. The MMSE score correlated with CRI-Total (r = 0.59, $p < 0.001$), CRI-Education (r = 0.54, $p < 0.001$), CRI-Working activity (r = 0.39, p = 0.003), and CRI-Leisure time (r = 0.29, p = 0.033) scores. The CDR-SOB score was correlated with CRI-Total (r = -0.40, p = 0.003), CRI-Education (r = -0.29, p = 0.031), and CRI-Working activity (r = -0.44, p = 0.001) scores.

After correction for multiple comparisons, the correlation between CRIq scores and some comprehensive neuropsychological test results failed to remain significant, except for the correlation between CRI-Leisure activity with verbal learning test recognition (rho = 0.35, p = 0.009), RCFT copy (rho = 0.38, p = 0.004), naming ability (r = 0.35, p = 0.008), and phonemic fluency test (r = 0.35, p = 0.009). The correlation between CRI-Total score and psychiatric symptoms failed to survive the correction for multiple comparisons. Correlation analyses data for each group are shown in **Supplementary Tables S2**, **S3** in **Supplementary Material**.

Regression Analyses on the Association Between CR and Cognitive Functions

The results of our regression analyses revealed the effect of CR on neuropsychological functions that were significant according to our correlation analyses (**Table 4**). CRI-Total (B = 3.00, p = 0.005), CRI-Education (B = 3.39, p = 0.002), and CRI-Leisure time (B = 2.56, p = 0.015) were prognostic for MMSE after adjusting for sex, diagnostic group, and CDR-SOB. No CR score was prognostic for CDR-SOB after adjusting for sex, diagnostic group, and MMSE. CRI-Leisure time significantly predicted naming ability (B = 2.20, p = 0.033). **Figure 1** shows the association between CRI-Total and MMSE and between CRI-Leisure time and naming ability. Regression analyses data for each group are shown in **Supplementary Tables S4**, **S5** in **Supplementary Material**.

Multivariable Regression Model

We used a multivariable regression model to test the relationship among CRI-Education, CRI-Working activity, CRI-Leisure time, and the composite cognitive score calculated from the comprehensive neuropsychological test. The results were then presented as a path diagram. The association between education and occupation is well known (Ng and Feldman, 2009; Nucci et al., 2012; Choi et al., 2016). Hence, we hypothesized the correlation between education and working activity and added the covariance between CRI-Education and CRI-Working activity in the model, which showed an adequate fit (χ^2 = 2.45, RMSEA = 0.064, CFI = 0.973).

Figure 2 graphically displays a significant regression estimate of CRI-Leisure time on the composite cognitive score (β = 0.32,

TABLE 2 | Cognitive reserve, neuropsychological, and psychiatric function of the participants.

	SCD (n = 36)		MCI (n = 19)		t or Z, p
	Mean ± SD	Min, max	Mean ± SD	Min, max	
Cognitive reserve index					
CRI-Education	58.42 ± 12.12	33, 84	58.32 ± 11.64	36, 84	−0.27, 0.790
CRI-Working activity	117.28 ± 15.24	101, 160	110.95 ± 13.31	101, 146	−2.10, 0.036*
CRI-Leisure time†	127.19 ± 10.39	107, 144	125.47 ± 7.08	115, 140	0.65, 0.521
CRI-Total†	101.22 ± 11.18	78, 124	97.53 ± 10.42	82, 118	1.19, 0.238
Comprehensive neuropsychological test					
Digit span forward†	−0.06 ± 0.98	−1.59, 2.18	−0.07 ± 1.21	−2.08, 2.24	0.05, 0.958
Digit span backward†	−0.30 ± 0.94	−2.06, 1.76	−0.39 ± 1.33	−3.47, 1.91	0.27, 0.789
SVLT, immediate recall†	0.47 ± 0.74	−0.84, 1.83	−0.15 ± 1.00	−1.98, 2.07	2.57, 0.013*
SVLT, delayed recall	0.27 ± 1.00	−1.87, 1.87	−0.64 ± 1.29	−2.96, 2.08	−2.72, 0.007*
SVLT, recognition	0.61 ± 0.74	−1.33, 1.65	−0.48 ± 1.75	−3.83, 1.68	−1.99, 0.046*
RCFT, copy	0.13 ± 0.69	−1.45, 1.24	−1.65 ± 2.59	−10.19, 0.49	−3.34, 0.001**
K-BNT †	0.02 ± 0.68	−1.40, 1.05	−1.03 ± 1.26	−2.98, 1.42	4.05, < 0.001***
COWAT, animal †	−0.18 ± 1.06	−2.85, 1.65	−0.76 ± 0.82	−2.36, 0.99	2.09, 0.042*
COWAT, phonemic †	−0.13 ± 0.79	−2.11, 1.20	−0.69 ± 0.82	−1.79, 0.61	2.45, 0.018*
Stroop test, color/word †	0.20 ± 0.85	−1.35, 1.74	−0.42 ± 1.20	−2.83, 1.33	2.24, 0.029*
TMT-A	0.27 ± 0.67	−2.32, 1.12	−2.08 ± 4.43	−14.19, 1.19	−2.75, 0.006**
TMT-B	−0.36 ± 1.25	−4.76, 0.96	−2.60 ± 2.40	−8.49, 0.48	−3.82, <0.001***
Psychiatric symptoms					
PANAS-P (SR: 10–50)†	19.44 ± 7.19	9, 40	17.68 ± 6.68	11, 34	0.88, 0.380
PANAS-N (SR: 10–50)	19.03 ± 6.42	11, 35	20.53 ± 8.71	12, 39	-0.33, 0.743
K-AES (SR: 18–72)†	50.89 ± 10.12	28, 70	47.79 ± 6.86	35, 58	1.20, 0.237
QOL-AD (SR: 13–52)	32.78 ± 7.64	23, 64	31.16 ± 3.85	26, 39	−0.36, 0.723
GDepS (SR: 0–30)†	13.86 ± 6.63	0, 29	14.42 ± 7.59	4, 29	−0.28, 0.778

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. † Normal distribution. Independent t test for continuous variables with normal distribution and Mann–Whitney U test for continuous variables without normal distribution were used. Comprehensive neuropsychological test results are presented as z-scores adjusted for age and years of education. SCD, subjective cognitive decline; MCI, mild cognitive impairment; SD, standard deviation; min, minimum; max, maximum; CRI, cognitive reserve index; SVLT, Seoul verbal learning test; RCFT, Rey-Osterrieth complex figure test; K-BNT, Korean version of the Boston naming test; COWAT, controlled oral word association test; TMT, trail making test; PANAS-P, positive and negative affect schedule-positive affect; PANAS-N, positive and negative affect schedule-negative affect; K-AES, Korean version of the apathy evaluation scale; QOL-AD, quality of life-Alzheimer's disease; GDepS, geriatric depression scale; SR, score range.*

p = 0.011). CRI-Education (β = -0.11, p = 0.441) and CRI-Working activity (β = 0.27, p = 0.053) did not show significant regression estimates on the composite cognitive score. Correlation between CRI-Education and CRI-Working activity (r = 0.45, p = 0.003 as shown in **Figure 2**) was found.

We also used a multivariable regression model to determine the association between CRI subdomains and cognitive subdomains. The result shows that CR-Leisure activity is predictive of cognitive subdomains such as language function (β = 0.29, p = 0.021), memory (β = 0.33, p = 0.009), visuospatial function (β = 0.29, p = 0.019), and frontal executive function (β = 0.26, p = 0.041), while CRI-Working activity predicts visuospatial function (β = 0.33, p = 0.015). CRI-Education failed to predict any cognitive domain. The results are presented in **Supplementary Figure S1** in the **Supplementary Material**.

DISCUSSION

In the present study, we hypothesized that the lifetime comprehensive CR may positively associate with neuropsychological function and psychiatric symptoms in patients at the early stage of cognitive decline. The results showed that total CR and its subdomains of education, working activity, and leisure time were associated with global cognitive function. In addition, only the CR based on leisure activity was associated with naming ability and the composite cognitive score in the early stage of cognitive decline, while education and working activity showed no association with detailed neuropsychological function and composite cognitive score.

As expected, we found a correlation between comprehensive CR and global cognition in patients at the early stage of cognitive decline. In the correlation analyses, the MMSE score, which reflects global cognition, was correlated with all CR proxies, including total CR, education, working activity, and leisure time scores, and its association with total CR, education, and leisure time remained significant in the regression analyses. These results are in line with previous research findings that a higher CR is associated with a higher cognition in global measures in healthy older adults (Liu et al., 2013; Lavrencic et al., 2018). Since old individuals with a high CR can respond flexibly to the beginning of cognitive decline due to aging or neurodegeneration (Scarmeas, 2007), our results in patients with SCD or MCI can support the CR theory that a higher CR, accumulated through

TABLE 3 | Correlation between cognitive reserve and neuropsychological function and psychiatric symptoms.

	CRI total†	CRI education	CRI working activity	CRI leisure time†
Global cognition				
MMSE	0.59, <0.001***	0.54, <0.001***	0.39, 0.003**	0.29, 0.033*
CDR-SOB	−0.40, 0.003**	−0.29, 0.031*	−0.44, 0.001**	−0.19, 0.157
Comprehensive neuropsychological test				
Digit span forward	−0.01, 0.941	−0.02, 0.864	0.00, 0.982	0.02, 0.876
Digit span backward †	−0.13, 0.353	−0.32, 0.015*	−0.06, 0.662	0.03, 0.814
SVLT, immediate recall †	0.22, 0.098	0.12, 0.367	0.04, 0.769	0.30, 0.026*
SVLT, delayed recall	0.23, 0.084	0.20, 0.146	0.15, 0.261	0.27, 0.044*
SVLT, recognition	0.25, 0.066	0.11, 0.435	0.14, 0.323	0.35, 0.009** ‡
RCFT, copy	0.21, 0.124	0.01, 0.968	0.18, 0.191	0.38, 0.004** ‡
K-BNT †	0.31, 0.019*	0.27, 0.044*	0.19, 0.169	0.35, 0.008** ‡
COWAT, animal †	0.03, 0.841	0.06, 0.678	0.02, 0.894	0.08, 0.539
COWAT, phonemic †	0.10, 0.465	−0.06, 0.654	0.05, 0.720	0.35, 0.009** ‡
Stroop test, color/word †	0.03, 0.846	−0.01, 0.923	−0.06, 0.671	0.12, 0.398
TMT-A	0.14, 0.294	0.01, 0.933	0.14, 0.294	0.10, 0.464
TMT-B	0.36, 0.006**	0.23, 0.083	0.22, 0.101	0.18, 0.174
Psychiatric symptoms				
PANAS-P †	0.23, 0.039*	0.17, 0.205	0.07, 0.634	0.26, 0.052
PANAS-N	0.06, 0.667	−0.00, 0.989	−0.03, 0.856	0.14, 0.294
K-AES †	0.32, 0.018*	0.15, 0.287	0.13, 0.355	0.23, 0.083
QOL-AD	0.22, 0.106	0.13, 0.356	0.04, 0.762	0.24, 0.080
GDepS †	−0.26, 0.052	−0.22, 0.097	−0.19, 0.172	−0.14, 0.317

**p < 0.05, **p < 0.01, ***p < 0.001. † Normal distribution. ‡ Survived post hoc tests for multiple comparisons by Benjamini–Hochberg's method. Data are Pearson's r coefficients (between variables with normal distribution) or Spearman's rho coefficients (correlation including variables without normal distribution) and p values. CRI, cognitive reserve index; MMSE, mini-mental state examination; CDR-SOB, clinical dementia rating-sum of boxes; SVLT, Seoul verbal learning test; RCFT, Rey-Osterrieth complex figure test; K-BNT, Korean version of the Boston naming test; COWAT, controlled oral word association test; TMT, trail making test; PANAS-P, positive and negative affect schedule-positive affect; PANAS-N, positive and negative affect schedule-negative affect; K-AES, Korean version of the apathy evaluation scale; QOL-AD, quality of life-Alzheimer's disease; GDepS, geriatric depression scale.*

a lifetime of brain stimulating activities, increases cognitive plasticity and delays the onset of MCI or dementia (Stern, 2002, 2012; Liu et al., 2013).

The associations between the subdomain scores of CR and the detailed neuropsychological tests representing the five cognitive domains are the main results of the present study. In the correlation analyses, the CRI-Total score correlated with the naming ability and executive function, which corresponds with previous results that a high level of CR was associated with the naming ability and divided attention (Puccioni and Vallesi, 2012; Cabral et al., 2016). We also found a correlation between educational attainment and naming ability. However, the effect of total CR and education disappeared after correction for multiple comparisons. It is well-established that there is a positive association between education and various cognitive functions, including memory, attention, and executive function in healthy older adults (Cabral et al., 2016; Opdebeeck et al., 2016; Zhang et al., 2019). However, other studies have also reported that education is associated with the level of cognitive performance in healthy older adults but not with the rate of cognitive decline itself (Bendayan et al., 2017; Seblova et al., 2020). Our result also showed little association between working activity and detailed neuropsychological tests in both correlation and regression analyses, implying little effect of working activities on detailed cognition at the early stage of cognitive decline. Previous studies have reported that although occupational attainment was a protective factor for cognitive decline in healthy older adults (Smart et al., 2014; Boots et al., 2015), it was a risk factor for progression from MCI to AD (Myung et al., 2017). Similarly, a large longitudinal study has suggested a paradoxical relationship where CR is associated with slower cognitive decline in normal old adults and MCI due to AD patients and rapid decline in AD dementia patients (van Loenhoud et al., 2019). We assume that the difference we observed in the association between subdomains of CR and detailed neuropsychological function might be attributable to the characteristics of our participants who were at the early stage of cognitive decline.

Another main finding is the association between leisure activity and detailed neuropsychological functions. The regression analyses revealed that only lifetime leisure activity was associated with preserved naming ability. Moreover, in the competitive multivariable regression model, the composite cognitive score based on the 12 neuropsychological subtests and the cognitive domain scores based on language function, memory, visuospatial function, and frontal executive function were explained by leisure activity, and not by education and working activity. The measures for CR used in previous studies have also included leisure activities, such as reading books, newspapers, magazines (Leon et al., 2014; Cabral et al., 2016), playing games, playing a musical instrument, or collecting

TABLE 4 | Multivariable linear regression analysis for cognitive reserve variables in predicting global and detailed neuropsychological function.

Independent variables	B	Standard error	β	*t*, *p*	Model fitness
Dependent variable: MMSE					
CRI-Total †	0.004	0.001	0.41	3.00, 0.005**	$F = 9.63$
Sex	0.003	0.03	0.01	0.08, 0.937	$p < 0.001$***
Diagnosis group	−0.07	0.03	−0.28	−2.08, 0.044*	$R^2 = 0.49$
CDR-SOB	−0.04	0.03	−0.21	−1.46, 0.153	Adj-$R^2 = 0.44$
CRI-Education	0.22	0.07	0.41	3.39, 0.002**	$F = 10.65$
Sex	−0.01	0.03	−0.04	−0.34, 0.738	$p < 0.001$***
Diagnosis group	−0.08	0.03	−0.33	−2.52, 0.016*	$R^2 = 0.52$
CDR-SOB	−0.04	0.02	−0.22	−1.54, 0.131	Adj-$R^2 = 0.47$
CRI-Working activity	0.10	0.17	0.11	0.61, 0.547	$F = 6.19$
Sex	−0.02	0.05	−0.06	−0.34, 0.738	$p = 0.001$**
Diagnosis group	−0.07	0.03	−0.28	−1.89, 0.066	$R^2 = 0.38$
CDR-SOB	−0.06	0.03	−0.34	−2.12, 0.035*	Adj-$R^2 = 0.32$
CRI-Leisure time	0.01	0.002	0.35	2.56, 0.015*	$F = 8.64$
Sex	−0.07	0.04	−0.30	−2.10, 0.042*	$p < 0.001$***
Diagnosis group	−0.06	0.03	−0.26	−1.89, 0.066	$R^2 = 0.46$
CDR-SOB	−0.03	0.03	−0.16	−1.01, 0.320	Adj-$R^2 = 0.41$
Dependent variable: CDR-SOB					
CRI-Total	−0.01	0.01	−0.16	−1.02, 0.312	$F = 7.68$
Sex	0.24	0.20	0.17	1.22, 0.231	$p < 0.001$***
Diagnosis group	0.42	0.19	0.30	2.18, 0.035*	$R^2 = 0.43$
MMSE	−1.39	0.95	−0.34	−1.46, 0.153	Adj-$R^2 = 0.38$
CRI-Education	−0.32	0.48	−0.10	−0.68, 0.500	$F = 7.436$
Sex	0.29	0.19	0.21	1.55, 0.130	$p < 0.001$***
Diagnosis group	0.44	0.20	0.32	2.20, 0.034*	$R^2 = 0.43$
MMSE	−1.51	0.98	−0.26	−1.54, 0.131	Adj-$R^2 = 0.37$
CRI-Working activity	0.02	0.96	0.004	0.02, 0.982	$F = 7.23$
Sex	0.32	0.25	0.23	1.28, 0.206	$p < 0.001$***
Diagnosis group	0.40	0.19	0.29	2.08, 0.044*	$R^2 = 0.42$
MMSE	−1.86	0.85	−0.32	−2.19, 0.035*	Adj-$R^2 = 0.36$
Dependent variable: SVLT recognition					
CRI-Leisure time	0.01	0.02	0.06	0.26, 0.797	$F = 1.36$
Sex	0.49	0.40	0.28	1.21, 0.235	$p = 0.275$
Diagnosis group	−0.28	0.37	−0.14	−0.75, 0.459	$R^2 = 0.17$
CDR-SOB	−0.54	0.37	−0.31	−1.47, 0.152	Adj-$R^2 = 0.04$
Dependent variable: RCFT copy					
CRI-Leisure time †	−0.03	0.03	−0.25	−0.83, 0.419	$F = 0.67$
Sex	−0.09	0.52	−0.05	−0.17, 0.870	$p = 0.623$
Diagnosis group	−0.66	0.58	−0.33	−1.15, 0.267	$R^2 = 0.14$
CDR-SOB	−0.16	0.45	−0.12	−0.35, 0.730	Adj-R^2 = -0.07
Dependent variable: K-BNT†					
CRI-Leisure time †	0.04	0.02	0.33	2.20, 0.033*	$F = 6.10$
Sex	−0.15	0.35	−0.07	−0.44, 0.662	$p = 0.001$**
Diagnosis group	−0.98	0.32	−0.45	−3.06, 0.004**	$R^2 = 0.38$
CDR-SOB	−0.001	0.27	−0.001	−0.01, 0.996	Adj-$R^2 = 0.32$

(Continued)

TABLE 4 | Continued

Independent variables	B	Standard error	β	*t*, *p*	Model fitness
Dependent variable: COWAT phonemic†					
CRI-Leisure time †	0.01	0.02	0.13	0.77, 0.448	$F = 1.67$
Sex	−0.08	0.32	−0.04	−0.24, 0.811	$p = 0.176$
Diagnosis group	−0.39	0.29	−0.23	−1.35, 0.186	$R^2 = 0.14$
CDR-SOB	−0.14	0.25	−0.11	−0.56, 0.581	Adj-$R^2 = 0.06$

$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. † Normal distribution. For each dependent variable, multivariable regression analysis was performed. Log transformation was performed for variables without normal distribution. Sex was coded as 1 (male) or 2 (female), and diagnostic group was coded as 0 (SCD) or 1 (MCI). MMSE, mini-mental state examination; CRI, cognitive reserve index; CDR-SOB, clinical dementia rating-sum of boxes; Adj-R^2, adjusted R^2; SVLT, Seoul verbal learning test; RCFT, Rey-Osterrieth complex figure test; K-BNT, Korean version of the Boston naming test; COWAT, controlled oral word association test; SCD, subjective cognitive decline; MCI, mild cognitive impairment.

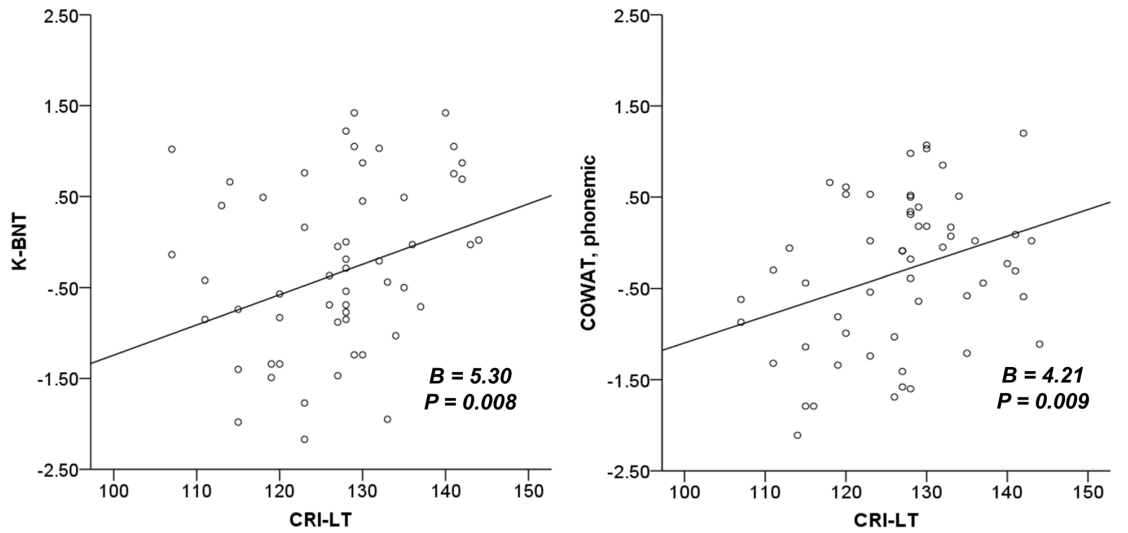

FIGURE 1 | The association between cognitive reserve and global cognition and naming ability. K-BNT is presented as *z*-score adjusted for age and years of education. MMSE, mini-mental state examination; CRI, cognitive reserve index; K-BNT, Korean version of the Boston naming test; CRI-LT, CRI-Leisure time.

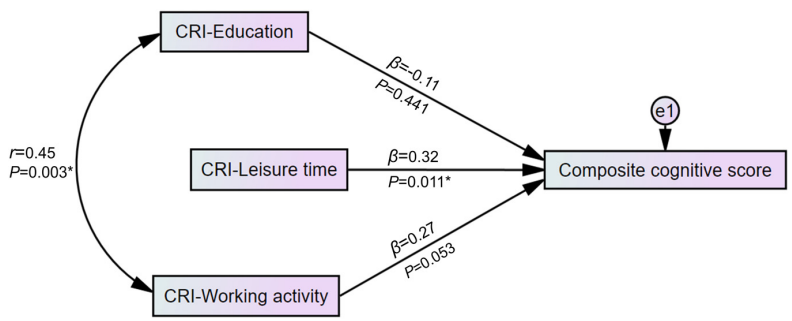

FIGURE 2 | Path diagram of the association between the domains of cognitive reserve and the composite cognitive score. Standardized scores of detailed neuropsychological tests were used to construct a composite cognitive score. In a multivariable regression model with an adequate fit ($\chi^2 = 2.45$, RMSEA = 0.064, CFI = 0.973), CRI-Leisure time showed a significant regression weight for the composite cognitive score. *$p < 0.05$. CRI, cognitive reserve index; e1, residual error variable; β, standardized regression weight; *r*, correlation estimate; RMSEA, root-mean-square error of approximation; CFI, Comparative Fit Index.

things (Leon et al., 2014). CR could reportedly enhance verbal memory, executive function, and attention in healthy older adults (Puccioni and Vallesi, 2012; Cabral et al., 2016). In our study, we used CRIq, which thoroughly measures lifetime leisure activity, and includes weekly activities, such as reading newspapers and magazines, sports, driving, dancing, and using new technologies; monthly activities, such as social gathering, going to cinema or theater, gardening, handicraft, taking care of children or elderly people, and playing instruments; and annual activities, such as watching concerts, attending conferences, going on overnight

trips, or the number of books read (Nucci et al., 2012). We believe that this comprehensive evaluation of lifetime leisure activity may have affected the significant relationship between CRI-Leisure time scores and cognitive function in naming ability and cognitive domain scores even in the beginning of cognitive decline. Leisure activities were consistently found to be protective against cognitive decline and incident dementia in healthy older adults (Akbaraly et al., 2009; Wang et al., 2012). In addition, the participants in our study were in the early stage of cognitive decline and CR might have been utilized as a compensatory mechanism in the aspects of everyday life (Tomaszewski Farias et al., 2018) and neural networks (Liang et al., 2011) against cognitive decline (Stern, 2012). The low education level in our study also may have contributed to the effect of leisure on cognitive function, because education has been reported to affect the association between leisure activity and cognitive function in a previous study, where an association between leisure activities and cognitive function was only observed in low-educated old adults (Park et al., 2019). We assume that lifetime leisure activities can help adapt to the early cognitive decline, particularly in naming ability and cognitive domains including language, memory, visuospatial, and frontal executive function through abstract and mental stimulating activities.

An association between CR and psychiatric symptoms was not found in our study. We assessed psychiatric symptoms that start to become more frequent in the early stage of dementia (Lyketsos et al., 2011), but failed to find any association between CR and apathy, affect, QOL, and depression. Apathy and a low positive affect are known to be the main behavioral and psychological symptoms of dementia commonly observed in various types of dementia (Gilley et al., 1991; Harrison et al., 2016; Breitve et al., 2018). Lower mood, motivation, activity, and affect can be manifested as the prodromal or initial symptoms in the dementia continuum (van Dalen et al., 2018; Tay et al., 2020). Apathy is also known to persist during the course of the disease (van der Linde et al., 2016) and is highly associated with impaired cognitive function (Reichman et al., 1996; Brown and Pluck, 2000; Breitve et al., 2018). In this context, a highly accumulated CR may help maintain the motivation and positive affect through various experiences and learnings over a lifetime at the very beginning of dementia. However, CR did not show a correlation with apathy and positive effect in this study after correction of multiple comparisons for psychiatric symptoms. This inconsistency may be attributed to the small size of our study leading to a low statistical power. Larger studies in the future may find significant associations.

We note several strengths and limitations to our study. The strength of the present study is in the characteristics of the participants who were at the early stage of cognitive decline and the measurement of CR and neuropsychological function. The most comprehensive validated measure for CR (Kartschmit et al., 2019) and the five domains of cognition evaluated in our study enabled us to determine that only leisure activity predicted cognitive decline in participants in early stage of cognitive decline. Popular and well-validated screening tests including functional ability and psychiatric symptoms are also other strengths of our study. However, the small sample size and low education level in our participants can limit the generalizability of our results. In addition, although we initially aimed to recruit participants at the pre-dementia stage, the sample was relatively heterogeneous, including those who were diagnosed with MCI and SCD. Due to the lack of information on AD biomarker evaluation, the type of dementia, i.e., AD, vascular dementia, dementia of Lewy body, and subcortical vascular dementia, was also not determined. Although SCD can be a potential risk factor for MCI or dementia, the majority of individuals with SCD were cognitively stable in longitudinal studies (Hessen et al., 2017; Lee et al., 2020). In addition, the neuropsychological symptoms of dementia also differ based on the type of dementia. Hence, the participants in our study can represent various neuropsychological profiles, which can be another limitation. Despite the lack of dementia biomarker evaluation, such as brain imaging or a CSF study, experienced clinicians in our study confirmed the diagnosis clinically based on the DSM-5 (American Psychiatric Association, 2013), Petersons' criteria (Petersen, 2004), and research criteria of SCD (Jessen et al., 2014) and effectively excluded participants with dementia; other major psychiatric disorders, such as major depressive disorder and anxiety disorders; and neurologic diseases, such as Parkinson's disease, epilepsy, and cerebrovascular disease.

In conclusion, we found an association between comprehensive CR and global cognition in patients at the early stage of cognitive decline. In particular, the lifetime leisure activity predicted naming ability and cognitive functions in domains including language, memory, visuospatial, and frontal executive function. These results may highlight the mental and social stimulation of leisure activity on maintaining cognition levels at the beginning of cognitive decline. Future studies should include a large number of participants with normal cognition, SCD, MCI, and dementia to compare the association between comprehensive CR and cognition across the diagnostic groups in the continuum of dementia. Additional evaluation of the biomarkers of cognitive decline would identify the neural mechanism of CR in aging and neurodegeneration.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request from the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Gachon University Gil Medical Center (Approval number: GAIRB2019-230). The participants provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

S-JC, J-YL, and JK designed the study. SL, JK, DK, SW, and S-JC acquired the data. SL, JK, and DK analyzed the data. SL and JK

wrote the manuscript. SW, J-YL, and S-JC critically reviewed the manuscript for important intellectual content. All authors reviewed and approved the final version of the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.590607/full#supplementary-material

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Age- and Sex-Specific Prevalence and Modifiable Risk Factors of Mild Cognitive Impairment Among Older Adults in China: A Population-Based Observational Study

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Background: Minimal data are available on the prevalence of mild cognitive impairment (MCI) in older Chinese adults. Moreover, the current information on MCI shows important geographical variations.

Objective: We aimed to assess the prevalence and risk factors for MCI by age and sex among older adults in a North Chinese population.

Methods: In this population-based cross-sectional study, we enrolled a random sample of 4,943 adults aged ≥ 60 years between March 2018 and June 2019 in Tianjin, China. Of these, 312 individuals were excluded due to a lack of data (e.g., fasting blood test). As a result, 4,631 subjects were assessed. Individuals with MCI were identified using neuropsychological assessments, including the Mini-Mental State Examination and Activities of Daily Living scale, based on a modified version of the Petersen's criteria.

Results: The mean (SD) age of the 4,631 participants was 67.6 (4.89) years, and 2,579 (55.7%) were female. The overall age- and sex-standardized prevalence of MCI in our study population was 10.7%. There were significant associations of MCI with age [65–69 vs. 60–64 years, OR = 0.74; 95% confidence interval (CI): 0.58, 0.96], physical activity (≥23.0 vs. <23.0 MET-hours/week, OR = 0.79; 95% CI: 0.64, 0.96), body mass index (BMI) (OR = 0.92; 95% CI: 0.89, 0.95), grip strength (OR = 0.50; 95% CI: 0.38, 0.67), hypertension (yes vs. no, OR = 1.44; 95% CI: 1.18, 1.77), higher levels of sleepiness (OR = 1.80; 95% CI: 1.36, 2.37), and longer sleep duration (OR = 1.40; 95% CI: 1.14, 1.72). The inverse association between BMI and MCI was stronger in older age groups (*P* for heterogeneity = 0.003). Moreover, the magnitude of association between triglycerides and MCI was different between the sexes (*P* for heterogeneity = 0.029).

Conclusion: The age- and sex-standardized prevalence of MCI was 10.7% in the study sample. Physical activity, BMI, grip strength, sleepiness, sleep duration, and hypertension were associated with the prevalence of MCI. Additionally, triglycerides and BMI might be differently associated with the presence of MCI for different sexes and age stages, respectively.

Keywords: mild cognitive impairment, prevalence, risk factors, sex differences, age differences, older adults

INTRODUCTION

Dementia is a common geriatric illness that is characterized by a decline in cognition that inhibits daily function and places a significant burden on patients, families, and social care systems (Langa, 2018, Cognitive Aging). Mild cognitive impairment (MCI) is a transitional state between normal aging and dementia, and approximately 10–20% of MCI patients progress annually to dementia (Winblad et al., 2004; Subramanyam and Singh, 2016). While there is no effective treatment available for the MCI-to-dementia progression, the burden of the disease can be reduced through primary prevention.

In recent decades, the rapid growth of the elderly population in China has spurred research interest in the cause and prevention of MCI. Previous epidemiological studies have demonstrated that sociodemographic, lifestyle, and vascular factors may be associated with MCI risk (Chiam et al., 2004; Lee et al., 2009; Qiu et al., 2010; Mohan et al., 2019). Since aging and certain hormonal changes (e.g., estrogen) involve a heightened susceptibility to cognitive decline, the association between lifestyle and MCI may vary depending on age and sex. However, few studies have examined the age- and sex-related differences in risk factors for MCI (House et al., 1988; Williams and Umberson, 2004). Our literature review only identified one cross-sectional study from China that analyzed the association between modifiable risk factors and MCI stratified by sex (Zhang et al., 2019). However, that study had a small sample size, lacked a formal test for heterogeneity, and only examined a limited number of predictors. Unlike other countries where no sex differences in the prevalence of MCI have been observed (Au et al., 2017), data from China have shown that the prevalence of MCI is higher in females than males (Nie et al., 2011). Additionally, a meta-analysis revealed that there was a difference in the prevalence of MCI between North and South China, but detailed data regarding the prevalence of MCI in Northern China have remained sparse (Nie et al., 2011).

In this study, we investigated the sex and age differences in the prevalence of MCI and the association between multiple influencing factors and MCI stratified by age and sex in a large-scale cross-sectional study in Northern China. This study should help to gain a better understanding of MCI and strategies to protect older people against cognitive decline.

MATERIALS AND METHODS

Study Population

This cross-sectional analysis used data collected at baseline from the Tianjin Elderly Nutrition and Cognition Cohort study (Clinical Trials Registration Identifier: ChiCTR2000034348), an ongoing elderly population-based prospective cohort study focused on the relationship between nutrition and cognition in China. Briefly, participants were recruited from the Baodi area of Tianjin, China. All participants had sufficient mobility, vision, and hearing to complete the assessments, and were aged 60 years or older at enrollment between March 2018 and June 2019. Using multistage cluster sampling, we randomly selected three communities in the Baodi District. From the three communities, we identified a total of 5,577 eligible subjects. Those who were willing to participate underwent a thorough clinical examination, personal interview, and cognitive function assessment, administered by licensed physicians, trained graduate students, and psychologists, respectively (n = 4,943; participation rate = 88.6%). Subjects who did not undergo a fasting blood test (n = 7), had a history of cerebrovascular disease (n = 91), cancer (n = 37), severe mental illness (n = 29), Parkinson's disease (n = 9), and Alzheimer's disease (AD) (n = 4) were excluded. As such, 4,766 subjects were included in the dataset (mean [standard deviation (SD)] age: 67.6 (4.89) years; males, 44.3%) (**Figure 1**). The study protocols were approved by the Ethics Committee of Tianjin Medical University, China (approval/protocol number: TMUhMEC2018013), and all participants provided their written informed consent before participating in the study. If a participant was illiterate, then informed consent was sought from their legal representative. All experimental procedures adhered strictly to the study protocol.

Definition of Mild Cognitive Impairment

We used a modified version of the Petersen's criteria (Petersen, 2004) to diagnose MCI: (1) subjective memory complaints over at least 6 months, preferably corroborated by an informant; (2) a Mini-Mental State Examination (MMSE) score of $\leq$17 points for illiterate participants, $\leq$20 points for those with primary school education, and $\leq$24 points for those with secondary education or above (Katzman et al., 1988); (3) absence of dementia (Diagnostic and Statistical Manual of Mental Disorders-IV criteria), AD (National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association criteria), psychiatric disorders, cerebral damage, or physical diseases leading to cognitive impairment; (4) cognitive performance indicated by 1.5 SD below the age-corrected (and education, where available) norms on at least one test in the neuropsychological battery (Ritchie et al., 2001); and (5) no/minimal impairment of daily life activities, as measured by the Activities of Daily Living Scale (ADL) ($<$26 points) (Perneczky et al., 2006). MCI patients had to fulfill the above five criteria, and the diagnosis of MCI was based

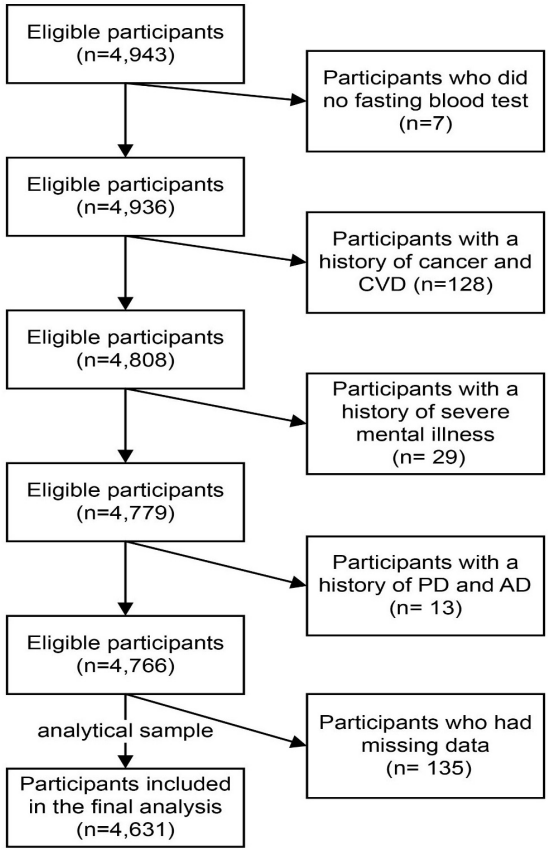

FIGURE 1 | Flow diagram showing the selection of the study population. CVD, cerebrovascular disease; PD, Parkinson's disease; AD, Alzheimer's disease.

on an expert consensus by a panel of physicians, neurologists, neuropsychologists, and psychiatrists.

Measures

We focused specifically on potential risk factors for MCI, including sociodemographic (e.g., sex and age) and health-related variables (e.g., lifestyle and physical performance). These variables were collected via a face-to-face interview or clinical examination according to a structured protocol.

The sociodemographic characteristics included age (60–64, 65–69, 70–74, and ≥75 years), sex, education level (illiterate, primary school, or middle school; high school, and above), household income (<3,000, 3,000–5,000, and >5,000 RMB), employment status (working full or part-time; not working or retired), marital status (married; single, divorced, or widowed).

Lifestyle variables included smoking, alcohol drinking, physical activity, and sleep characteristics. Smoking status was grouped by current smoker, ex-smoker, or never smoked. Drinking status was grouped as current drinker or non-drinker. Physical activity (PA) was measured using a short version of the International Physical Activity Questionnaire, which collects information on the number of minutes spent on vigorous-intensity activities, moderate-intensity activities, walking, and sitting during the past week (Craig et al., 2003). Total PA, expressed in metabolic equivalent hours per week (MET-h/week), was calculated by multiplying the hours per week of vigorous, moderate, and walking activities with their corresponding MET coefficients (8.0, 4.0, and 3.3, respectively) and then summing the scores (Craig et al., 2003). The level of total PA was divided into two categories: <23 and ≥23 METs-h/week (Cao, 2015). Self-reported sleep characteristics were derived from participants' sleep duration and daytime sleepiness. Sleep duration was assessed by asking the question: "How many hours do you usually sleep at night?" Self-reported sleep duration was examined in categories of short sleep duration (<6.5 h) and long sleep duration (>8.5 h), with 6.5–8.5 h of sleep as the reference (Keage et al., 2012; Chiu et al., 2016). Besides, the eight-item Epworth Sleepiness Scale was used to assess the likelihood of falling asleep in common daily situations (Johns, 1992). Each item scored on a four-point scale was summed with scores ranging from 0 to 24, with higher scores indicating greater sleepiness. The scale has well-established validity and reliability (Johns, 1992).

Physical performance was assessed by grip strength (GS). GS was measured using an electronic handheld dynamometer (EH101; CAMRY, Guangdong, China). Participants were tested by trained technicians under the same conditions. Forces were measured twice for each hand, and the greatest force was used for the analyses. Additionally, GS relative to body weight (kg/kg) was also calculated because of the involvement of body weight in the maximal performance of muscle strength (Jimenez-Pavon et al., 2012; Huang et al., 2014a,b).

Clinical examinations included a general physical examination and biochemical blood tests. Height and body weight were measured using a standard protocol, and the body mass index (BMI) was calculated as weight/height^2 (kg/m^2). We used a BMI cutoff of 24 based on the Working Group on Obesity in China and the standard of WS/T 428-2013 (China) recommendations for country-specific and ethnicity-specific BMI cutoff in China (Zhou, 2002). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice in the right arm using an automatic device (KD598; Andon) after minutes of rest in a seated position. The mean of these two measurements was taken as the final blood pressure (BP). Hypertension was defined as having a BP higher than 140/90 mm Hg (SBP/DBP) or a history of hypertension. Blood samples for the assessment of fasting blood glucose (FBG) and blood lipids were drawn from the antecubital vein, with the participants in a seated position. Specimens were collected in siliconized vacuum plastic tubes. The FBG levels were measured using the glucose oxidase method. Diabetes was defined as having an FBG of ≥7.0 mmol/L, oral glucose tolerance test value of ≥11.1 mmol/L, HbA1c of ≥48 mmol/mol (6.5%), or a history of diabetes, which is in accordance with the latest recommendations from the American Diabetes Association (American Diabetes Association, 2014). As for plasma lipids, plasma triglycerides (TG) and total cholesterol (TC) were measured using enzymatic methods. Plasma low-density

lipoprotein cholesterol (LDL-C) was quantified using the polyvinyl sulfuric acid precipitation method, and serum high-density lipoprotein cholesterol (HDL-C) was measured using the chemical precipitation method and appropriate kits (Roche Cobas 8000 modular analyzer, Mannheim, Germany). Hyperlipidemia was defined as TC of ≥5.17 mmol/L, TG of ≥1.7 mmol/L, LDL-C of ≥3.37 mmol/L, or a history of hyperlipidemia.

Statistical Analysis

Age and sex were specified as sociodemographic risk factors of interest *a priori*. We applied the age- and sex-specific rates of MCI of the China and Tianjin standard populations, obtained from the China Health Statistical Yearbook 2018, to calculate sex- and age-standardized prevalence estimates so that direct comparisons could be made between populations. Associations between the sociodemographic variables, health-related variables, and MCI were examined overall and by age and sex. For the descriptive analysis, an analysis of variance for continuous variables (except for weight-adjusted GS by an analysis of covariance) and a logistic regression analysis for categorical variables were used to compare differences between those with and without MCI. Continuous variables are presented as the geometric mean and 95% confidence interval (CI) after logarithmic transformation. Categorical variables are shown as a number (percentage). For the main analysis, the status of MCI was considered as a dependent variable, and the sociodemographic and health-related variables as independent variables. Continuous variables (e.g., sleepiness and GS) were log-transformed before analysis, except for BMI. Associations between the sociodemographic and health-related variables and the status of MCI were assessed using a multivariate logistic regression in two different models, where the odds ratios (ORs) and 95% CIs were calculated. Specifically, model 1 was adjusted for age and sex, while model 2 was additionally adjusted for all other variables, including education level, income, marital status, employment status, PA, smoking, alcohol drinking, sleep duration, sleepiness, BMI, GS, hypertension, diabetes, and hyperlipidemia. The *P*-values for linear trends were calculated by treating the categorical variables as ordinal variables in the model. We used χ^2 likelihood-ratio tests to assess the heterogeneity within age and sex categories. General linear models were used to calculate β coefficients and 95% CIs for risk factors related to the MMSE scores in two different models: (1) adjusting for age and sex, (2) additionally adjusting for all other variables.

For all predefined variables, missing values represented less than 3% of the data of each variable. Information pertinent to the physical examination, including BMI, BP, blood glucose, and blood lipids, was missing for 135 participants. Compared with the participants included in the analytical sample (n = 4,631), which is the population who satisfied the inclusion and/or exclusion criteria and were included in the statistical analysis, those excluded due to missing data (n = 135) did not differ in terms of sex but were younger and more likely to have lower education levels (**Supplementary Table 1**). A complete case analysis was conducted as the main analysis, and multiple imputations were performed for missing data in a sensitivity analysis. Multivariate normal imputation was used to impute missing physical examination values (Lee and Carlin, 2010). Moreover, variance inflation factors (VIFs) were used to detect multicolinearity among covariates in the final model. VIFs exceeding 10 were a sign of multicolinearity. To assess the potential for reverse causation, we conducted sensitivity analyses by (1) excluding 362 participants who had changed their lifestyles including diet, drinking, smoking, PA, and sleeping habits, in the past 5 years; (2) excluding 766 participants with long-term medication use; and (3) using a conceptual framework (Price et al., 2018) to categorize the risk factors for MCI into distal factors (e.g., age, sex, education level, income, marital status, and employment), lifestyle behavioral risk factors, and proximal factors (e.g., BMI, GS, and hypertension), assuming the former influenced the latter. This framework determined the factors to retain in the multivariate models. We treated the distal factors as potential confounders of the association between lifestyle behavioral risk factors and the prevalence of MCI. Similarly, the distal and lifestyle behavioral risk factors were considered as potential confounders for the association between proximal risk factors and MCI (**Figure 2**). SAS version 9.4 (SAS Institute, Inc., Cary, NC, United States) was used for all statistical analyses. A two-sided *P*-value of <0.05 was considered statistically significant.

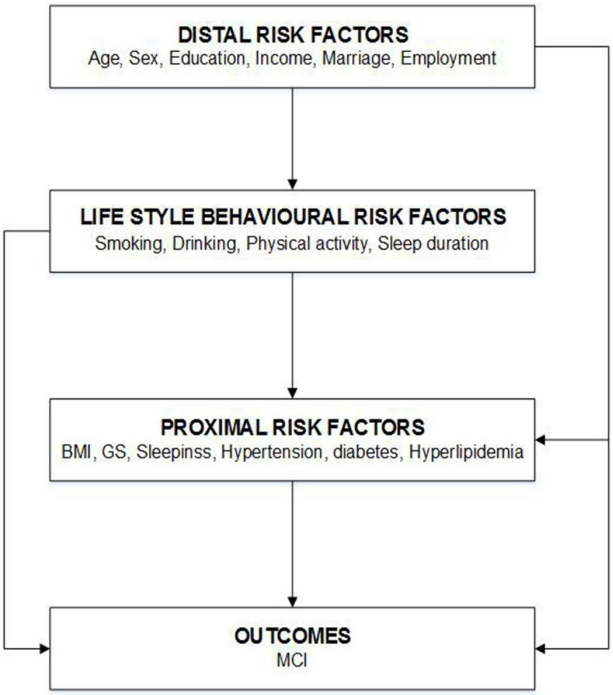

FIGURE 2 | Conceptual model risk factors for mild cognitive impairment (MCI).

TABLE 1 | Prevalence of (MCI) in adults aged 60 years and older by age group and sex.

Group	*n*	MCI	Crude prevalence (%)	Standardized prevalence (%)[a]	
				Tianjin	China
Participants	4,631	468	10.1	10.7	10.7
Sex					
Males	2,052	162	7.9	8.2	8.2
Females	2,579	306	11.9	13.0	13.1
Age (years)					
60–64	1,464	141	9.6	9.5	9.5
65–69	1,633	132	8.1	7.9	7.9
70–74	1,069	127	11.9	11.8	11.7
75-∼	465	68	14.6	15.1	15.1

[a]*Age- and sex-standardized prevalence.*

RESULTS

Study Participant Characteristics

Between March 2018 and June 2019, a total of 4,631 individuals were recruited (2,052 males and 2,579 females), of whom 468 (10.1%) were diagnosed with MCI (162 males and 306 females, **Table 1**). Participants were divided into four age groups, 60–64, 65–69, 70–74, and ≥75 years, with 141 (9.6%), 132 (8.1%), 127 (11.9%), and 68 (14.6%) people, respectively. When standardized to China's national population, the age- and sex-standardized prevalence of MCI among people aged ≥ 60 years was 10.7% in the study sample. The age-standardized MCI prevalence was slightly higher in females (13.1%) than in males (8.2%). Overall, the sex-standardized MCI prevalence increased with age, with prevalence rates in each age category at 9.5, 7.9, 11.7, and 15.1%, respectively. The prevalence increased more steeply with age in females than males (**Figure 3A**). Participants with below high school level education had a consistently higher prevalence of MCI than those with an education level of high school or above in all age groups. Meanwhile, the MCI prevalence increased with increasing age in participants with an education level below high school (**Figure 3B**).

Participants' characteristics are summarized in **Table 2**. The majority of patients with MCI were female. MCI patients tended to be older and have lower levels of education and income, while non-MCI participants were more likely to be married and working full- or part-time ($P < 0.05$). Regarding health-related variables, a greater proportion of MCI patients had hypertension, longer sleep duration, higher LDL-C, SBP, sleepiness, lower BMI, and GS, and were less likely to be physically active, drinkers, and ex-smokers ($P < 0.05$). Not surprisingly, participants who exhibited MCI displayed a worse cognitive performance on the MMSE ($P < 0.0001$). The descriptive profile of the participants showed some sex and age differences (**Tables 3**, **4**). Males with MCI were more likely to have hypertension and less likely to be married than females ($P < 0.05$). Compared to males, females with MCI had lower education levels, income, BMI, a higher proportion of longer sleep duration, were less likely to have hyperlipidemia and a full- or part-time job, but were more likely to be older and have higher levels of sleepiness ($P < 0.05$). Participants with MCI tended to have lower GS and MMSE scores in all four age groups ($P < 0.05$).

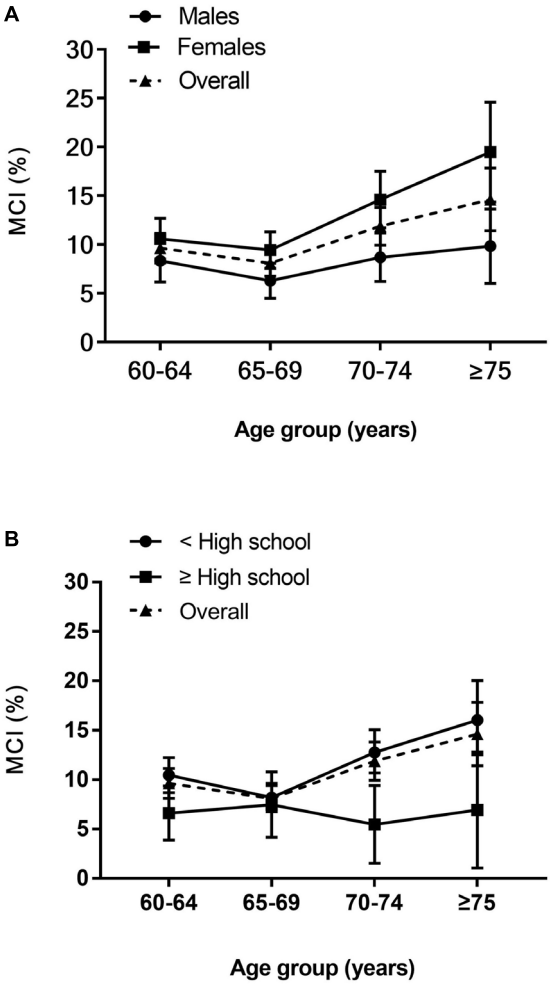

FIGURE 3 | Age-specific prevalence of MCI. **(A)** Age and sex-specific prevalence of MCI. **(B)** Age and education-specific prevalence of MCI. Prevalence point estimates are presented with (vertical) 95% CIs.

Association Between the Sociodemographic Variables and MCI

After multivariate adjustment, participants in the second age group (65–69 years) were significantly associated with MCI when compared with individuals aged 60–64 years (OR = 0.74; 95% CI: 0.58, 0.96; **Table 5**). No significant association was seen between other sociodemographic variables and MCI. Intriguingly, in the youngest age group, participants who worked full- or part-time appeared to have a lower prevalence of MCI relative to those who were retired or not working (OR = 0.40; 95% CI: 0.17, 0.84; **Figure 4B**).

TABLE 2 | Characteristics of the participants with and without MCI (n = 4,631).

Characteristics	MCI		*P*-value[a]
	No	Yes	
No. of subjects	4,163	468	
Sociodemographic characteristics			
Age group (%, years)[c]			
60–64	1,323 (31.8)	141 (30.1)	0.47
65–69	1,501 (36.1)	132 (28.2)	**<0.001**[d]
70–74	942 (22.6)	127 (27.1)	**0.029**
≥75	387 (9.54)	68 (14.5)	**<0.001**
Sex (males, %)	1,890 (45.4)	162 (34.6)	**<0.0001**
Education level (≥high school, %)	707 (17.0)	51 (10.9)	**<0.001**
Currently married (%)	3,673 (88.2)	394 (84.2)	**0.012**
Income status (%, RMB)			
<3,000	2,992 (71.9)	363 (77.6)	**<0.01**
3,000–5,000	592 (14.2)	61 (13.0)	0.48
>5,000	579 (13.9)	44 (9.40)	**<0.01**
Employed (%)	287 (6.89)	19 (4.06)	**0.021**
Health-related variables			
BMI (kg/m^2)	25.6 (25.5, 25.7)[b]	25.0 (24.7, 25.3)	**<0.001**
Hypertension (%)	2,166 (52.0)	282 (60.3)	**<0.001**
SBP (mmHg)	132.4 (132.0, 132.8)	134.6 (133.4, 135.8)	**<0.001**
DBP (mmHg)	80.5 (80.3, 80.8)	80.5 (79.9, 81.2)	1.00
Diabetes (%)	761 (18.3)	89 (19.0)	0.70
FBG (mmol/L)	5.33 (5.30, 5.37)	5.22 (5.11, 5.32)	**0.028**
Hyperlipidemia (%)	2,654 (63.8)	278 (59.4)	0.064
TC (mmol/L)	5.09 (5.06, 5.12)	5.12 (5.02, 5.22)	0.59
TG (mmol/L)	1.41 (1.39, 1.43)	1.35 (1.29, 1.41)	0.070
LDL-C (mmol/L)	2.48 (2.45, 2.50)	2.55 (2.49, 2.62)	**0.019**
HDL-C (mmol/L)	1.28 (1.27, 1.29)	1.29 (1.27, 1.32)	0.34
GS (adjusted weight) (kg)	23.1 (22.8, 23.3)	20.4 (19.7, 21.2)	**<0.0001**
PA (≥23.0 METs-h/w, %)	2,700 (64.9)	265 (56.6)	**<0.001**
Sleepiness (scores)	9.33 (9.24, 9.43)	10.0 (9.73, 10.3)	**<0.0001**
Sleep duration (%)			
<6.5 h	263 (6.32)	23 (4.91)	0.23
6.5–8.5 h	2,266 (54.4)	202 (43.2)	**<0.0001**
>8.5 h	1,634 (39.3)	243 (51.9)	**<0.0001**
Smoking status (%)			
Non-smoker	2,648 (63.6)	316 (67.5)	0.095
Ex-smoker	335 (8.05)	21 (4.49)	**<0.01**
Current smoker	1,180 (28.3)	131 (28.0)	0.87
Alcohol drinking (%)	3,191 (23.4)	390 (16.7)	**<0.01**
MMSE scores	25.9 (25.8, 26.1)	17.8 (17.5, 18.1)	**<0.0001**
ADL scores	15.4 (15.3, 15.6)	15.6 (15.3, 15.9)	0.34

BMI, body mass index; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; GS, grip strength; PA, physical activity; METs, metabolic equivalents; MMSE, Mini-Mental State Examination; ADL, activities of daily living.

[a] *Analysis of variance or logistic regression analysis.*

[b] *Geometric least square mean (95% confidence interval) (all such values).*

[c] *Categorical variables are expressed as number (percentage) of participants.*

[d] *Boldface indicates statistical significance ($P < 0.05$) (or appropriate value).*

Association Between Health-Related Variables and Mild Cognitive Impairment

In the present study, BMI was inversely associated with MCI in both the adjusted models, where the ORs (95% CI) were 0.95 (0.92, 0.98) and 0.92 (0.89, 0.95), respectively (**Table 5**). Similar associations were observed when males and females were analyzed separately (P for heterogeneity = 0.50; **Figure 4A** and **Supplementary Table 4**). Moreover, the magnitude of the inverse association between BMI and MCI increased with age (P for heterogeneity = 0.003; **Figure 4B** and **Supplementary Table 5**). Additionally, when we modeled BMI as a categorical variable, the OR (95% CI) of developing MCI was 0.68 (0.55, 0.85) for participants with a BMI of ≥24 kg/m^2 compared to those with a BMI of <24 kg/m^2. We found that hyperlipidemia was significantly associated with a lower prevalence of MCI in females only in the stratified analyses by sex and age (OR = 0.70; 95% CI: 0.54, 0.91). Moreover, the magnitude of the association between TG and MCI was different between the sexes (P for heterogeneity = 0.016). In this study, GS (OR = 0.50; 95% CI: 0.38, 0.67) and PA (OR = 0.79; 95% CI: 0.64, 0.96) levels were negatively associated with the presence of MCI in all models. In contrast, the presence of hypertension (OR = 1.44; 95% CI: 1.18, 1.77) was positively associated with the prevalence of MCI in all models (**Table 5**). As for the age- and sex-specific associations, the GS–MCI relationship in all age groups and the associations of MCI with PA and hypertension in the older age group (70–74 years) remained statistically significant after multivariate adjustment ($P < 0.05$; **Figure 4B**). GS and hypertension were significantly associated with MCI in both sexes ($P < 0.05$; **Figure 4A**). The prevalence of MCI increased with higher levels of sleepiness (OR = 1.80; 95% CI: 1.36, 2.37) and sleep duration (>8.5 h vs. 6.5 to 8.5 h) (OR = 1.40; 95% CI: 1.14, 1.72) (**Table 5**). The former association remained significant in both sexes and the younger age group (60–69 years), and the latter association remained significant in females and the older age group (65–74 years) after multivariate adjustment (P for heterogeneity > 0.05; **Figures 4A,B**).

Association Between Multivariate Factors and the MMSE Score

In the final multivariate models, those with a higher education level, higher income level, married, and currently working had an increase in the MMSE score from 0.55–1.52 as per unit increase of the respective variable ($P < 0.05$).

In contrast, the cognitive scores tended to decrease, ranging from 0.69 to 1.48 among females and those in the older age groups ($P < 0.05$; **Table 6**). For the abovementioned health-related variables, those with a higher BMI, higher GS, and hyperlipidemia showed an increase of 0.47 to 2.91 on average in the cognitive scores ($P < 0.05$). In contrast, smokers, those with hypertension, higher levels of sleepiness, and higher sleep duration showed a decrease of 0.27–0.85 on average in the cognitive scores ($P < 0.05$). All VIFs ranged from 1.03 to 2.54, indicating no colinearity

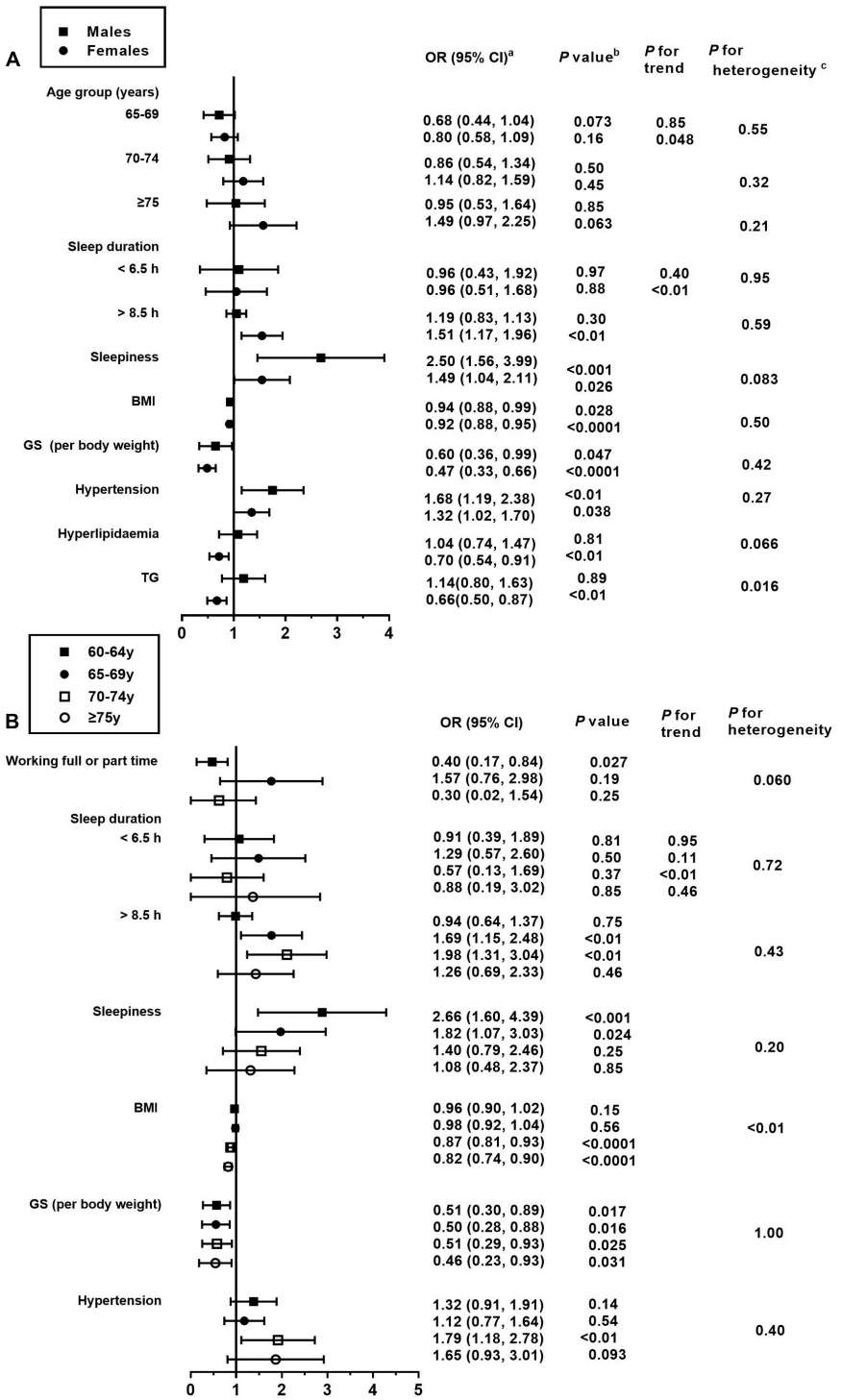

FIGURE 4 | Association between the risk factors and MCI, stratified by sex and age. BMI, body mass index; GS, grip strength; TG, triglyceride. This figure only shows the risk factors that are significantly associated with MCI. For details on the association of all risk factors with MCI, please refer to **Supplementary Tables 3**, **4**. (a) Fully adjusted models are adjusted for age, sex, education level, income status, marital status, employment status, physical activity, smoking status, drinking status, sleep duration, BMI, GS, hypertension, diabetes, hyperlipidemia or its diagnostic indicators, and sleepiness. (b) Obtained using a multiple logistic regression analysis. (c) Comparison between the odds ratios associated with sex **(A)** and age groups **(B)** using *P* for heterogeneity.

TABLE 3 | Characteristics of the participants with and without MCI stratified by sex (n = 4,631).

Characteristics	Males (n = 2,052)		P-value[a]	Females (n = 2,579)		P-value
	No	Yes		No	Yes	
No. of subjects	1,890	162		2,273	306	
Sociodemographic characteristics						
Age group (%, years)[c]						
60–64	572 (30.3)	52 (32.1)	0.63	751 (33.0)	89 (29.1)	0.17
65–69	656 (34.7)	44 (27.2)	0.053	845 (37.2)	88 (28.8)	**<0.01**
70–74	451 (23.9)	43 (26.5)	0.44	491 (21.6)	84 (27.5)	**0.021**
≥ 75	211 (11.2)	23 (14.2)	0.24	186 (8.18)	45 (14.7)	**<0.001**
Education level (≥ high school,%)	478 (25.3)	35 (21.6)	0.30	229 (10.1)	16 (5.23)	**<0.01**
Currently married (%)	1,704 (90.2)	136 (84.0)	**0.014**[d]	1,969 (86.6)	258 (84.3)	0.27
Income status (%, RMB)						
<3,000	1,259 (66.6)	113 (69.8)	0.42	1,733 (76.2)	250 (81.7)	**0.034**
3,000–5,000	295 (15.6)	29 (17.9)	0.44	297 (13.1)	32 (10.5)	0.20
>5,000	336 (17.8)	20 (12.4)	0.082	243 (10.7)	24 (7.8)	0.13
Employed (%)	206 (10.9)	15 (9.26)	0.52	81 (3.56)	4 (1.31)	**0.047**
Health-related variables						
BMI (kg/m^2)	25.4 (25.3, 25.6)[b]	25.1 (24.7, 25.6)	0.19	25.7 (25.5, 25.8)	24.9 (24.6, 25.3)	**<0.001**
Hypertension (%)	954 (50.5)	102 (63.0)	**<0.01**	1,212 (53.3)	180 (58.8)	0.070
SBP (mmHg)	133.1 (132.5, 133.7)	134.6 (132.7, 136.6)	0.14	131.9 (131.3, 132.4)	134.6 (133.1, 136.1)	**<0.001**
DBP (mmHg)	81.4 (81.0, 81.7)	81.9 (80.7, 83.0)	0.43	79.9 (79.6, 80.2)	79.9 (79.0, 80.7)	0.98
Diabetes (%)	319 (16.9)	30 (18.5)	0.59	442 (19.5)	59 (19.3)	0.95
FBG (mmol/L)	5.33 (5.28, 5.38)	5.21 (5.04, 5.37)	0.18	5.34 (5.30, 5.39)	5.22 (5.10, 5.35)	0.078
Hyperlipidemia (%)	994 (52.6)	85 (52.5)	0.98	1,660 (70.0)	193 (63.1)	**<0.001**
TC (mmol/L)	4.85 (4.80, 4.90)	4.87 (4.71, 5.04)	0.77	5.30 (5.26, 5.35)	5.26 (5.13, 5.38)	0.49
TG (mmol/L)	1.24 (1.21, 1.27)	1.26 (1.17, 1.36)	0.66	1.57 (1.54, 1.60)	1.40 (1.32, 1.47)	**<0.0001**
LDL-C (mmol/L)	2.36 (2.33, 2.39)	2.43 (2.33, 2.54)	0.15	2.58 (2.55, 2.61)	2.62 (2.54, 2.70)	0.32
HDL-C (mmol/L)	1.26 (1.24, 1.28)	1.24 (1.19, 1.30)	0.52	1.30 (1.29, 1.31)	1.32 (1.29, 1.36)	0.12
GS (adjusted weight) (kg)	31.3 (30.8, 31.7)	28.9 (27.6, 30.3)	**<0.0001**	18.1 (17.8, 18.3)	16.0 (15.4, 16.6)	**<0.0001**
PA(≥ 23.0 METs-h/w,%)	1,235 (65.3)	91 (56.2)	**0.020**	1,465 (64.5)	174 (56.9)	**<0.01**
Sleepiness (scores)	9.32 (9.18, 9.45)	10.4 (9.85, 10.9)	**<0.0001**	9.35 (9.22, 9.48)	9.85 (9.49, 10.22)	**<0.01**
Sleep duration (%)						
<6.5 h	121 (6.40)	9 (5.56)	0.67	142 (6.25)	14 (4.58)	0.25
6.5–8.5 h	1,072 (56.7)	79 (48.8)	0.051	1,194 (52.5)	123 (40.2)	**<0.0001**
>8.5 h	697 (36.9)	74 (45.7)	**0.027**	937 (41.2)	169 (55.2)	**<0.0001**
Smoking status (%)						
Non-smoker	707 (37.4)	57 (35.2)	0.57	1,941 (85.4)	259 (84.6)	0.73
Ex-smoker	286 (15.1)	16 (9.88)	0.075	50 (2.20)	5 (1.63)	0.52
Current smoker	898 (47.5)	89 (54.9)	0.071	282 (12.4)	42 (13.7)	0.51
Alcohol drinking (%)	981 (48.1)	92 (43.2)	0.23	2,210 (2.77)	298 (2.61)	0.87
MMSE scores	27.2 (27.1, 27.3)	20.7 (20.4, 21.1)	**<0.0001**	24.8 (24.6, 25.0)	16.4 (16.1, 16.8)	**<0.0001**
ADL scores	15.2 (15.0, 15.3)	15.0 (14.5, 15.5)	0.40	15.7 (15.5, 15.8)	16.0 (15.5, 16.4)	0.19

BMI, body mass index; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; GS, grip strength; PA, physical activity; METs, metabolic equivalents; MMSE, Mini-Mental State Examination; ADL, activities of daily living.

[a] *Analysis of variance or logistic regression analysis.*

[b] *Geometric least square mean (95% confidence interval) (all such values).*

[c] *Categorical variables are expressed as number (percentage) of participants.*

[d] *Boldface indicates statistical significance ($P < 0.05$) (or appropriate value).*

issues. Similar results were also observed in the sensitivity analyses (**Table 7** and **Supplementary Table 2**). However, the positive association of female sex and older age with MCI appeared enhanced in multivariate models determined by the conceptual framework (**Table 8**). In the analysis where multiple imputations were used to impute missing values, the results are marginally unchanged (**Supplementary Table 3**).

TABLE 4 | Characteristics of the participants with and without MCI among the different age groups (n = 4,631)[a].

Characteristics	60–64 (n = 1,464)		65–69 (n = 1,633)		70–74 (n = 1,069)		≥75 (n = 465)	
	No	Yes	No	Yes	No	Yes	No	Yes
No. of subjects	1,323	141	1,501	132	942	127	397	68
Sociodemographic characteristics								
Sex (males,%)[d,e,f,g]	751 (43.2)	89 (36.9)	845 (43.7)	88 (33.3)	491 (47.9)	84 (33.9)	186 (53.2)	45 (33.8)
Education level (≥ high school,%)[c,e]	296 (22.4)	21 (14.9)	223 (14.9)	18 (13.6)	121 (12.9)	7 (5.51)	67 (16.9)	5 (7.35)
Currently married (%)[e]	1,210 (91.5)	126 (89.4)	1,352 (90.1)	122 (92.4)	814 (86.4)	100 (78.7)	297 (74.8)	46 (67.7)
Income status (%, RMB)								
<3,000[e]	917 (69.3)	106 (75.2)	1,091 (72.7)	91 (68.9)	677 (71.9)	109 (85.8)	307 (77.3)	57 (83.8)
3,000–5,000	197 (14.9)	23 (16.3)	210 (14.0)	21 (15.9)	140 (14.86)	12 (9.45)	45 (11.34)	5 (7.35)
>5,000[c,e]	209 (15.8)	12 (8.51)	200 (13.3)	20 (15.2)	125 (13.3)	6 (4.72)	45 (11.3)	6 (8.82)
Employed (%)[c]	152 (11.5)	7 (4.96)	100 (6.66)	11 (8.33)	28 (2.97)	1 (0.79)	7 (1.76)	0 (0)
Health-related variables								
BMI (kg/m^2)[e,f]	25.6 (25.4, 25.8)[b]	25.4 (24.9, 26.0)	25.6 (25.5, 25.8)	25.7 (25.2, 26.3)	25.6 (25.3, 25.8)	24.6 (24.0, 25.1)	25.2 (24.9, 25.6)	23.6 (22.9, 24.3)
Hypertension (%)[e]	601 (45.4)	74 (52.5)	814 (54.2)	77 (54.2)	536 (56.9)	88 (69.3)	215 (54.2)	43 (63.2)
SBP (mmHg)[e]	130.2 (129.5, 130.8)	131.6 (129.5, 133.7)	132.6 (132.0, 133.3)	133.8 (131.5, 136.0)	134.4 (133.6, 135.2)	138.0 (135.7, 140.4)	134.6 (133.4, 135.8)	136.2 (133.3, 139.3)
DBP (mmHg)	80.5 (80.1, 80.9)	80.3 (79.1, 81.5)	80.7 (80.4, 81.1)	80.9 (79.7, 82.2)	80.7 (80.2, 81.2)	80.6 (79.3, 82.0)	79.8 (79.1, 80.4)	80.1 (78.5, 81.8)
Diabetes (%)	224 (16.9)	29 (20.6)	275 (18.3)	29 (22.0)	188 (20.0)	25 (19.7)	74 (18.6)	6 (8.82)
FBG (mmol/L)[e,f]	5.29 (5.24, 5.35)	5.28 (5.11, 5.47)	5.34 (5.29, 5.40)	5.40 (5.21, 5.60)	5.38 (5.30, 5.45)	5.10 (4.91, 5.30)	5.34 (5.24, 5.45)	4.95 (4.72, 5.19)
Hyperlipidemia (%)[c]	882 (66.7)	82 (58.2)	949 (63.2)	85 (64.4)	576 (61.2)	75 (59.1)	247 (62.2)	36 (52.9)
TC (mmol/L)	5.13 (5.07, 5.19)	5.11 (4.93, 5.30)	5.06 (5.00, 5.11)	5.27 (5.07, 5.47)	5.10 (5.03, 5.17)	5.04 (4.85, 5.23)	5.08 (4.98, 5.19)	5.01 (4.77, 5.28)
TG (mmol/L)	1.44 (1.40, 1.48)	1.35 (1.24, 1.46)	1.44 (1.40, 1.47)	1.45 (1.33, 1.58)	1.36 (1.32, 1.40)	1.30 (1.19, 1.42)	1.33 (1.27, 1.39)	1.26 (1.13, 1.41)
LDL-C (mmol/L)	2.50 (2.46, 2.54)	2.57 (2.46, 2.69)	2.45 (2.41, 2.48)	2.56 (2.44, 2.69)	2.48 (2.44, 2.53)	2.54 (2.43, 2.66)	2.48 (2.42, 2.55)	2.53 (2.37, 2.71)
HDL-C (mmol/L)	1.29 (1.28, 1.31)	1.29 (1.24, 1.33)	1.26 (1.24, 1.27)	1.29 (1.24, 1.35)	1.30 (1.27, 1.32)	1.31 (1.24, 1.38)	1.28 (1.25, 1.31)	1.29 (1.22, 1.37)
GS (adjusted weight) (kg)[c,d,e,f]	24.9 (24.4, 25.5)	22.5 (21.1, 24.0)	23.1 (22.7, 23.5)	20.5 (19.3, 21.9)	21.5 (21.0, 22.1)	19.3 (18.0, 20.7)	21.0 (20.2, 21.9)	18.0 (16.2, 20.0)
PA (≥23.0 METs-h/w, %)[c,e]	881 (66.6)	82 (58.2)	984 (65.6)	80 (60.6)	600 (63.7)	64 (50.4)	235 (59.2)	39 (57.4)
Sleepiness (scores)[c,d]	9.35 (9.18, 9.51)	10.5 (9.96, 11.1)	9.29 (9.14, 9.45)	9.97 (9.43, 10.5)	9.45 (9.25, 9.65)	9.77 (9.22, 10.4)	9.18 (8.89, 9.49)	9.60 (8.87, 10.4)
Sleep duration (%)								
<6.5 h	85 (6.42)	8 (5.67)	103 (6.86)	9 (6.82)	55 (5.84)	3 (2.36)	20 (5.04)	3 (4.41)
6.5–8.5 h[d,e]	779 (58.9)	81 (57.5)	858 (57.2)	59 (44.7)	459 (48.7)	38 (29.9)	170 (42.8)	24 (35.3)
>8.5 h[d,e]	459 (34.7)	52 (36.9)	540 (36.0)	64 (48.5)	428 (45.4)	86 (67.7)	207 (52.1)	41 (60.3)
Smoking status (%)								
Non-smoker	839 (63.4)	92 (65.3)	955 (63.6)	87 (65.9)	601 (63.8)	89 (70.1)	253 (63.7)	48 (70.6)
Ex-smoker[f]	88 (6.65)	7 (4.96)	120 (7.99)	7 (5.30)	87 (9.24)	6 (4.72)	40 (10.1)	1 (1.47)
Current smoker	396 (29.9)	42 (29.8)	426 (28.4)	38 (28.8)	254 (27.0)	32 (25.2)	104 (26.2)	19 (27.9)
Drinking status (Yes, %)[d]	983 (25.7)	112 (20.6)	1,166 (22.3)	113 (14.4)	733 (22.2)	108 (15.0)	309 (22.2)	57 (16.2)
MMSE scores[c,d,e,f]	26.2 (26.0, 26.4)	18.4 (17.9, 18.8)	25.7 (25.5, 25.9)	18.2 (17.6, 18.7)	26.0 (25.7, 26.3)	17.5 (17.0, 18.1)	25.2 (24.74, 25.7)	16.6 (15.8, 17.4)

(Continued)

TABLE 4 | Continued

Characteristics	60–64 (*n* = 1,464)		65–69 (*n* = 1,633)		70–74 (*n* = 1,069)		≥75 (*n* = 465)	
	No	Yes	No	Yes	No	Yes	No	Yes
ADL scores	15.1 (14.9, 15.3)	15.1 (14.7, 15.6)	15.4 (15.2, 15.6)	15.7 (15.1, 16.4)	15.6 (15.4, 15.8)	15.6 (15.0, 16.3)	16.5 (16.0, 17.0)	16.3 (15.2, 17.4)

BMI, body mass index; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; GS, grip strength; PA, physical activity; METs, metabolic equivalents; MMSE, Mini-Mental State Examination; ADL, Activities of Daily Living.

[a] *Analysis of variance or logistic regression analysis.*

[b] *Geometric least square mean (95% confidence interval) (all such values).*

[c] *$P < 0.05$ for group of 60 to 64 years;*

[d] *$P < 0.05$ for group of 65 to 69 years;*

[e] *$P < 0.05$ for group of 70–74 years;*

[f] *$P < 0.05$ for group of aged 75 and over.*

[g] *Categorical variables are expressed as number (percentage) of participants.*

DISCUSSION

We observed that the age- and sex-standardized prevalence of MCI in people living in Northern China was 10.7%. Also, our results revealed that certain sociodemographic and health-related characteristics were associated with the prevalence of MCI. This study may have implications for the development of MCI prevention and healthy aging policy.

The prevalence of MCI observed in this study was similar (10.7%) to that previously reported in other regions of China (9.7–16.5%) (Nie et al., 2011). Consistent with the sex-specific prevalence shown in a meta-analysis from China (Nie et al., 2011), the current study demonstrated a higher prevalence of MCI in females than in males. In addition, the overall pattern of MCI prevalence across different age groups in this study was similar to the results of a meta-analysis (Nie et al., 2011), where the MCI prevalence was much higher and continued to increase after 70 years of age, suggesting that age-related changes likely play a greater role in MCI after 70 years. Although many studies calculated the prevalence of MCI, they were limited in terms of having a small sample size, limited number of risk factors assessed, and lack of stratified analysis. Therefore, more extensive and in-depth analyses of the association between risk factors and the prevalence of MCI are warranted.

Earlier studies found an association between MCI and sociodemographic characteristics, including age, sex, education level, income status, and marital status in older adults (Rawtaer et al., 2017; Vanoh et al., 2017; Tsoy et al., 2019; Fernandez-Blazquez et al., 2020). However, we found that most of the recognized risk factors for MCI were not different between those with and without MCI. However, employment status in the youngest group and age distribution were significantly different between those with and without MCI in the sample analyzed. There are inconsistencies between studies regarding the association between age and MCI (Vanoh et al., 2017; Hussin et al., 2019; Kume et al., 2019). In this analysis, we excluded participants with cerebrovascular disease, AD, and severe limitations in ADL, leading to fewer MCI cases in the 65–69 age group. This may explain our observations that participants in the second age stratum (65–69 years) had a significantly lower prevalence of MCI when compared to people aged 60–64 years. In addition, the results of this study also showed that the higher age group (≥70 years) was associated with lower MMSE scores regardless of their ADL scores. It has been suggested that the prevalence of MCI was significantly lower for those aged less than 70 years, demonstrating an association between age and MCI in this study. In the sensitivity analysis, the oldest age group and females were found to be associated with an increased prevalence of MCI. However, the association disappeared after adjusting for the proximal variables, which indicated that proximal factors might mediate the association of age and sex with MCI. Therefore, the proximal factors (i.e., BMI, GS, sleepiness, hypertension, diabetes, and hyperlipidemia) were considered as modifiable factors that can be targeted to reduce the prevalence of MCI. Few studies have focused on the association between employment status in people aged over 60 years and MCI (Mohan et al., 2019). Going to work results in more opportunities to go out and participate in social activities that are potential protective factors for MCI (Anderson et al., 2012; Gao et al., 2018). The employment status of Chinese over 60 years of age gradually changes after retirement. Similarly, in this study, as age increased, the number of adults having jobs gradually declined. Almost all of the participants in the oldest age group were retired. This could explain why the working status in the youngest group was positively associated with cognitive function in this study. Although no association was observed between other sociodemographic variables and the prevalence of MCI, these variables, including sex, education level, income status, and marital status, were associated with cognitive function scores. Therefore, these variables may be essential in informing strategies to improve cognitive function.

We found that participants with lower levels of PA, BMI, and GS, higher levels of sleepiness, longer sleep duration, and hypertension were associated with a higher prevalence of MCI. These findings are in accordance with previous studies (Babkoff et al., 1991; Sofi et al., 2011; Keage et al., 2012; Roberts and Knopman, 2013; Beydoun et al., 2014; Pearson et al., 2016; Liang et al., 2019; Liu et al., 2020; Pacifico et al., 2020). However, participants with MCI tended

TABLE 5 | Association between the risk factors and MCI (adjusted ORs and 95% confidence intervals; *n* = 4,631).

Variables	Age and sex adjusted		Multivariable adjusted[b]	
	OR (95%CI)	*P*-value[a]	OR (95%CI)	*P*-value
Sociodemographic characteristics				
Age group (years)				
60–64	1.00 (reference)	–	1.00 (reference)	–
65–69	0.83 (0.64, 1.06)	0.13	**0.74 (0.58, 0.96)**	**0.021**
70–74	1.29 (1.00, 1.66)	0.052	1.00 (0.76, 1.30)	0.98
≥75	**1.67 (1.22, 2.27)**	**<0.01[c]**	1.20 (0.86, 1.67)	0.28
***P* for trend[a]**	**<0.001**		0.25	
Females vs. males	**1.61 (1.32, 1.97)**	**<0.0001**	1.11 (0.83, 1.49)	0.47
High school and above	**0.68 (0.49, 0.92)**	**0.014**	0.81 (0.58, 1.13)	0.23
Income status (RMB)				
<3,000	1.00 (reference)	–	1.00 (reference)	–
3,000–5, 000	0.90 (0.67, 1.19)	0.46	1.14 (0.84, 1.53)	0.39
>5,000	**0.69 (0.49, 0.94)**	**0.024**	0.94 (0.65, 1.33)	0.71
***P* for trend**	**0.02**		>0.99	
Married vs. unmarried	0.81 (0.62, 1.07)	0.12	0.87 (0.67, 1.16)	0.34
Working vs. no work	0.72 (0.43, 1.13)	0.17	0.69 (0.41, 1.10)	0.14
Health-related variables				
PA (METs-h/w) (≥23.0 vs. <23.0**)**	**0.72 (0.60, 0.88)**	**<0.01**	**0.79 (0.64, 0.96)**	**0.018**
Smoking status				
Non-smoker	1.00 (reference)	–	1.00 (reference)	–
Ex-smoker	0.69 (0.42, 1.10)	0.13	0.65 (0.39, 1.03)	0.077
Current smoker	1.20 (0.94, 1.52)	0.15	1.21 (0.94, 1.56)	0.14
***P* for trend**	0.16		0.15	
Alcohol drinking	0.86 (0.64, 1.16)	0.32	0.85 (0.62, 1.16)	0.31
Sleep duration (h)				
<6.5 h	0.98 (0.61, 1.50)	0.91	0.94 (0.58, 1.46)	0.83
6.5–8.5 h	1.00 (reference)	–	1.00 (reference)	–
>8.5 h	**1.56 (1.28, 1.90)**	**<0.0001**	**1.40 (1.14, 1.72)**	**<0.01**
***P* for trend**	**<0.001**		**0.006**	
Sleepiness (scores)	**1.86 (1.41, 2.44)**	**<0.0001**	**1.80 (1.36, 2.37)**	**<0.0001**
BMI (kg/m^2)	**0.95 (0.92, 0.98)**	**<0.001**	**0.92 (0.89, 0.95)**	**<0.0001**
GS(per body weight) (kg/kg)	**0.54 (0.41, 0.71)**	**<0.0001**	**0.50 (0.38, 0.67)**	**<0.0001**
Hypertension	**1.37 (1.12, 1.67)**	**<0.01**	**1.44 (1.18, 1.77)**	**<0.001**
Diabetes	1.03 (0.80, 1.31)	0.79	1.01 (0.78, 1.30)	0.92
Hyperlipidemia	**0.75 (0.62, 0.92)**	**<0.01**	0.83 (0.67, 1.02)	0.070

BMI, body mass index; GS, grip strength; PA, physical activity; METs, metabolic equivalents.
[a] *Obtained by using multiple logistic regression analysis.*
[b] *Additionally adjusted for education level, income status, marriage status, employment status, physical activity, smoking status, drinking status, sleep duration, BMI, GS, hypertension, diabetes, hyperlipidemia, sleepiness.*
[c] *Boldface indicates statistical significance (P < 0.05) (or appropriate value).*

to be non-drinkers. This may contradict previous research findings (Mira et al., 2019). One possible reason is that participants with MCI who tended to be older and have hypertension may, in turn, change their lifestyle (e.g., quit drinking). Therefore, after multivariate adjustments, the association between drinking status and MCI disappeared. Besides, several meta-analyses have found that low to moderate alcohol drinking is associated with better global cognition scores, whereas excessive alcohol intake elevates the risk of progression to dementia in people with MCI (Lao et al., 2020; Zhang et al., 2020). However, we did not investigate alcohol consumption among those with and without MCI. Further studies on the association between alcohol consumption and MCI is required.

In this study, a negative association between BMI as either a categorical or a continuous variable and MCI was observed. Moreover, the inverse association was strengthened with increased age, and BMI was also positively associated with cognitive function scores. Similarly, other cross-sectional studies reported that being overweight was linked to a decreased prevalence of cognitive impairment in Chinese and Indonesian elderly (Hou et al., 2019; Vidyanti et al., 2020). In contrast, several prospective studies that analyzed the effects of BMI trajectories from middle to old age on

TABLE 6 | Association of the risk factors and MMSE scores in the total population (n = 4,631).

Variables	Age and sex adjusted			Multivariable adjusted[b]		
	β (95% CI)	*SE*	*P*-value[a]	β (95% CI)	*SE*	*P* value
Sociodemographic characteristics						
Age group						
60–64	(reference group)	–	–	(reference group)	–	–
65–69	-0.25 (-0.53, 0.03)	0.14	0.075	0.07 (-0.20, 0.33)	0.14	0.61
70–74	−0.47 (−0.79, −0.16)	0.16	**<0.01**[c]	0.22 (−0.08, 0.52)	0.15	0.16
≥ 75	−1.57 (−1.98, −1.15)	0.21	**<0.0001**	−0.69 (−1.09, −0.28)	0.21	**<0.001**
Sex (females vs. males)	−2.67 (−2.89, −2.44)	0.12	**<0.0001**	−1.48 (−0.80, −1.16)	0.16	**<0.0001**
High school and above	2.33 (2.02, 2.64)	0.16	**<0.0001**	1.52 (1.19, 1.85)	0.17	**<0.0001**
Income status (RMB)						
<3,000	(reference group)	–	–	(reference group)	–	–
3,000–5,000	1.38 (1.05, 1.70)	0.17	**<0.0001**	0.74 (0.42, 1.07)	0.16	**<0.0001**
>5,000	2.19 (1.86, 2.53)	0.17	**<0.0001**	1.07 (0.72, 1.42)	0.18	**<0.0001**
Marital status (Unmarried vs. married)	0.91 (0.56, 1.26)	0.18	**<0.0001**	0.55 (0.22, 0.89)	0.17	**<0.01**
Employment status (Working vs. No work**)**	0.046 (-0.42, 0.52)	0.24	0.85	0.20 (−0.25, 0.64)	0.23	0.38
Health related variables						
PA (METs-h/w) (≥23.0 vs. <23.0**)**	0.70 (0.47, 0.94)	0.12	**<0.0001**	0.31 (0.08, 0.53)	0.12	**<0.01**
Smoking status						
Non-smoker	(reference group)	–	–	(reference group)	–	–
Ex-smoker	0.062 (−0.40, 0.52)	0.24	0.79	0.24 (−0.19, 0.68)	0.22	0.28
Current smoker	−0.45 (−0.73, −0.16)	0.15	**<0.001**	−0.35 (−0.63, −0.07)	0.14	**0.013**
Alcohol drinking	0.18 (−0.14, 0.51)	0.16	0.26	0.24 (−0.07, 0.55)	0.16	0.13
Sleep duration (h)						
<6.5 h	−0.26 (−0.73, 0.22)	0.24	0.30	−0.32 (−0.78, 0.14)	0.23	0.17
6.5–8.5 h	(reference group)	–	–	(reference group)	–	–
>8.5 h	−1.28 (−1.52, −1.05)	0.12	**<0.0001**	−0.85 (−1.08, −0.62)	0.12	**<0.0001**
Sleepiness (scores)	−0.95 (−1.30, −0.60)	0.18	**<0.0001**	−0.71 (−1.04, −0.38)	0.17	**<0.0001**
BMI (kg/m^2)	1.61 (0.70, 2.51)	0.46	**<0.001**	2.91 (1.99, 3.84)	0.47	**<0.001**
GS(per body weight) (kg/kg)	1.94 (1.60, 2.29)	0.18	**<0.0001**	1.99 (1.64, 2.34)	0.18	**<0.0001**
Hypertension	−0.34 (−0.57, −0.11)	0.12	**<0.01**	−0.27 (−0.49, −0.05)	0.11	**0.017**
Diabetes	0.05 (−0.24, 0.34)	0.15	0.74	−0.03 (−0.31, 0.26)	0.14	0.86
Hyperlipidemia	0.80 (0.56, 1.04)	0.12	**<0.0001**	0.47 (0.24, 0.70)	0.12	**<0.0001**

BMI, body mass index; GS, grip strength; PA, physical activity; METs, metabolic equivalents; MMSE, Mini-Mental State Examination.
[a]*Obtained by using generalized linear model.*
[b]*Additionally adjusted for education level, income status, marriage status, employment status, physical activity, smoking status, drinking status, sleep duration, BMI, GS, hypertension, diabetes, hyperlipidemia, sleepiness.*
[c]*Boldface indicates statistical significance ($P < 0.05$) (or appropriate value).*

cognitive function showed that deceleration of weight gain at older ages reflected early signs of cognitive impairment (Wagner et al., 2020; Bohn et al., 2020). However, elevated body weight in middle-age might reduce cognitive function (Singh-Manoux et al., 2012; Suemoto et al., 2015; Wagner et al., 2020; Bohn et al., 2020; Floud et al., 2020). A recent systematic review is in agreement with our findings that AD risk is decreased when BMI surpassed 27 kg/m^2 in later life, suggesting that the elderly could increase their body weight to combat dementia (Qu et al., 2020). Several biologic processes have explained that higher BMI in later life may be beneficial by increasing insulin-like growth factor I (IGF-1) levels as well as leptin hormone levels and estrogen production (Yamamoto and Kato, 1993; Harvey et al., 2006; Singh et al., 2006), all of which are associated with better cognitive performance (Power et al., 2011). Furthermore, after age stratification, it was found that the negative association between BMI and the prevalence of MCI was strengthened with age. Although specific mechanisms for this observation remain unclear, it could be related to factors in specific settings. For example, the aging process involves multiple psychosocial, behavioral, and physiological changes, which may partially explain the differential associations between BMI and MCI across different age stages. More in-depth studies are warranted to explain the association between higher BMI and cognitive function in later life.

The association between hyperlipidemia and the prevalence of MCI remains controversial (Xue et al., 2017; Chen et al., 2018). A review and meta-analysis revealed that midlife high total serum cholesterol was associated with an increased risk

TABLE 7 | Association between the risk factors and MCI after excluding participants who had changed their lifestyle in the past 5 years (adjusted ORs and 95% CIs; *n* = 4,242).

Variables	Age and sex adjusted		Multivariable adjusted[b]	
	OR (95%CI)	*P*-value[a]	OR (95%CI)	*P*-value
Sociodemographic characteristics				
Age group (years)				
60–64	1.00 (reference)	–	1.00 (reference)	–
65–69	0.81 (0.62, 1.04)	0.10	**0.73 (0.56, 0.94)**	**0.017**
70–74	1.24 (0.95, 1.61)	0.11	0.96 (0.73, 1.27)	0.78
≥75	**1.69 (1.22, 2.31)[c]**	**<0.01**	1.20 (0.85, 1.69)	0.29
***P* for trend[a]**	**< 0.001**		0.31	
Females vs. males	**1.67 (1.36, 2.07)**	**<0.0001**	1.18 (0.87, 1.60)	0.29
High school and above	**0.68 (0.49, 0.92)**	**0.016**	0.82 (0.57, 1.15)	0.25
Income status (RMB)				
<3,000	1.00 (reference)	–	1.00 (reference)	–
3,000–5,000	0.93 (0.69, 1.24)	0.63	1.19 (0.87, 1.60)	0.26
>5,000	**0.68 (0.48, 0.95)**	**0.029**	0.94 (0.64, 1.35)	0.74
***P* for trend**	**0.035**		0.90	
Married vs. unmarried	0.82 (0.62, 1.09)	0.16	0.88 (0.66, 1.18)	0.38
Working vs. no work	0.71 (0.42, 1.14)	0.18	0.68 (0.40, 1.10)	0.14
Health-related variables				
PA (METs × h/w) (≥23.0 vs. <23.0)	**0.76 (0.62, 0.93)**	**<0.01**	**0.81 (0.66, 1.00)**	**0.049**
Smoking status				
Non-smoker	1.00 (reference)	–	1.00 (reference)	–
Ex-smoker	0.63 (0.35, 1.06)	0.098	0.61 (0.34, 1.04)	0.087
Smoker	1.18 (0.92, 1.52)	0.19	1.21 (0.93, 1.57)	0.16
***P* for trend**	0.20		0.17	
Alcohol drinking	0.92 (0.67, 1.25)	0.58	0.92 (0.66, 1.26)	0.59
Sleep duration (h)				
<6.5 h	0.93 (0.57, 1.46)	0.76	0.90 (0.54, 1.42)	0.66
6.58.5 h	1.00 (reference)	–	1.00 (reference)	–
>8.5 h	**1.52 (1.24, 1.87)**	**<0.0001**	**1.37 (1.11, 1.70)**	**<0.01**
***P* for trend**	**<0.001**		**<0.01**	
Sleepiness (scores)	**1.79 (1.34, 2.39)**	**<0.0001**	**1.72 (1.28, 2.30)**	**<0.001**
BMI (kg/m^2)	**0.95 (0.92, 0.98)**	**<0.001**	**0.93 (0.89, 0.96)**	**<0.0001**
GS (per body weight) (kg/kg)	**0.51 (0.39, 0.68)**	**<0.0001**	**0.47 (0.35, 0.64)**	**<0.0001**
Hypertension	**1.35 (1.11, 1.66)**	**<0.01**	**1.44 (1.16, 1.78)**	**<0.001**
Diabetes	0.99 (0.76, 1.28)	0.96	0.98 (0.75, 1.27)	0.89
Hyperlipidemia	**0.71 (0.58, 0.88)**	**<0.01**	**0.79 (0.64, 0.98)**	**0.031**

BMI, body mass index; GS, grip strength; PA, physical activity; METs, metabolic equivalents.
[a]Obtained by using multiple logistic regression analysis.
[b]Additionally adjusted for education level, income status, marriage status, employment status, physical activity, smoking status, drinking status, sleep duration, BMI, GS, hypertension, diabetes, hyperlipidemia, sleepiness.
[c]Boldface indicates statistical significance (P < 0.05) (or appropriate value).

of MCI, AD, and cognitive decline in later life. However, high cholesterol in later life was not associated with MCI, AD, dementia, or cognitive decline (Anstey et al., 2017). Furthermore, similar to previous studies, our study found a

TABLE 8 | Association between the risk factors and MCI: age- and sex-adjusted as well as multivariate-adjusted risk factors (adjusted ORs and 95% CIs; n = 4,631).

Variables	Age and sex adjusted		Multiple factor adjusted	
	OR (95%CI)	*P*-value[a]	OR (95%CI)	*P*-value
Distal risk factors[b]				
Age group (years)				
60–64	1.00 (reference)	–	1.00 (reference)	–
65–69	0.83 (0.64, 1.06)	0.13	0.80 (0.62, 1.02)	0.075
70–74	1.29 (1.00, 1.66)	0.052	1.20 (0.93, 1.56)	0.17
≥75	**1.67 (1.22, 2.27)[e]**	**<0.01**	**1.52 (1.10, 2.08)**	**0.011**
***P* for trend[a]**	**<0.001**		**<0.01**	
Females vs. males	**1.61 (1.32, 1.97)**	**<0.0001**	**1.47 (1.20, 1.81)**	**<0.001**
High school and above	**0.68 (0.49, 0.92)**	**0.014**	0.74 (0.52, 1.02)	0.073
***P* for trend**			0.21	
Income status (RMB)				
<3,000	1.00 (reference)	–	1.00 (reference)	–
3,000–5,000	0.90 (0.67, 1.19)	0.46	0.95 (0.71, 1.27)	0.74
>5,000	**0.69 (0.49, 0.94)**	**0.024**	0.78 (0.54, 1.09)	0.16
***P* for trend**	**0.023**		0.18	
Married vs. unmarried	0.81 (0.62, 1.07)	0.12	0.83 (0.64, 1.10)	0.20
Working vs. No work	0.72 (0.43, 1.13)	0.17	0.69 (0.41, 1.08)	0.13
Life style behavior risk factors[c]				
PA (METs × h/w) (≥ 23.0 vs. <23.0)	**0.72 (0.60, 0.88)**	**<0.01**	**0.78 (0.64, 0.96)**	**0.015**
Smoking status				
Non-smoker	1.00 (reference)	–	1.00 (reference)	–
Ex-smoker	0.69 (0.42, 1.10)	0.13	0.66 (0.40, 1.05)	0.092
Current smoker	1.20 (0.94, 1.52)	0.15	1.20 (0.93, 1.53)	0.15
***P* for trend**	0.16		0.29	
Alcohol drinking	0.86 (0.64, 1.16)	0.32	0.84 (0.61, 1.13)	0.25
Sleep duration (h)				
<6.5 h	0.98 (0.61, 1.50)	0.91	0.95 (0.59, 1.47)	0.83
6.5–8.5 h	1.00 (reference)	–	1.00 (reference)	–
>8.5 h	**1.56 (1.28, 1.90)**	**<0.0001**	**1.48 (1.21, 1.81)**	**<0.01**
***P* for trend**	**<0.001**		**<0.01**	
Proximal risk factors[d]				
Sleepiness (scores)	**1.86 (1.41, 2.44)**	**<0.0001**	**1.80 (1.36, 2.37)**	**<0.0001**
BMI (kg/m^2)	**0.95 (0.92, 0.98)**	**<0.001**	**0.92 (0.89, 0.95)**	**<0.0001**
GS(per body weight) (kg/kg)	**0.54 (0.41, 0.71)**	**<0.0001**	**0.50 (0.38, 0.67)**	**<0.0001**

(Continued)

TABLE 8 | Continued

Variables	Age and sex adjusted		Multiple factor adjusted	
	OR (95%CI)	*P*-value[a]	OR (95%CI)	*P*-value
Hypertension	**1.37 (1.12, 1.67)**	**<0.01**	**1.44 (1.18, 1.77)**	**<0.001**
Diabetes	1.03 (0.80, 1.31)	0.79	1.01 (0.78, 1.30)	0.92
Hyperlipidemia	**0.75 (0.62, 0.92)**	**<0.01**	0.83 (0.67, 1.02)	0.070

BMI, body mass index; GS, grip strength; PA, physical activity; METs, metabolic equivalents.
[a]*Obtained by using multiple logistic regression analysis.*
[b]*Additionally adjusted for education level, income status, marriage status, employment status.*
[c]*Additionally adjusted for education level, income status, marriage status, employment status, physical activity, smoking status, drinking status, sleep duration.*
[d]*Additionally adjusted for education level, income status, marriage status, employment status, physical activity, smoking status, drinking status, sleep duration, BMI, GS, hypertension, diabetes, hyperlipidemia, sleepiness.*
[e]*Boldface indicates statistical significance ($P < 0.05$) (or appropriate value).*

non-significant negative association between hyperlipidemia and the prevalence of MCI in the elderly, although the association was further strengthened in the sensitivity analysis. Interestingly, when stratified according to sex, the associations between serum lipids and cognitive impairment were only prominent in older females. Similarly, in a study by Kim and Park (2017), hyperlipidemia was reported as a protective factor for MCI in females. Furthermore, among the four indicators causing hyperlipidemia (i.e., TC, TG, LDL-C, and HDL-C), only TG was related to the prevalence of MCI among older females. Moreover, there appeared to be heterogeneity in the association between TG and MCI by sex. However, only a few studies have analyzed the relationship between serum lipids and cognitive impairment, depending on sex. In contrast, a cross-sectional study in rural China on serum lipids revealed an inverse association between TG and the prevalence of MCI in middle-aged males and a positive association between LDL-C and MCI in older females (Zhao et al., 2019). The discrepancy between that study and our findings may be partially due to differences in sample size, dietary habits, and MCI diagnostic criteria. The mechanisms by which increased TG improves cognitive function could be due to the following: Low TG concentrations have been suggested to be correlated with brain inflammation, frailty, low nutrition levels, and low endogenous estrogen (Hu et al., 2003; Yasui et al., 2008). Estrogen may facilitate better cognitive function by exerting effects on specific brain regions such as the prefrontal cortex and hippocampus (Hara et al., 2015). This may explain the positive association between TG levels and cognitive function only in females. On the other hand, low TG levels may, in turn, reflect a low nutrition level implying pathological changes or be a marker for early cognitive impairment. However, potential mechanisms underlying the observed interactions between sex and blood lipid indicators with cognitive impairment are still unclear. Moreover, the reasons for this inconsistency in different sexes could partly be because cognitive impairment has quite distinct sex differences in terms of innate physiology, social behavior, and relevant factors (Ritchie et al., 2010). As this is a cross-sectional study, further prospective research is needed to explore the sex differences with the association of hyperlipidemia and its diagnostic indicators with MCI.

The ratio of males to females (1:1.26) and the education level among the Northern Chinese sample are in line with the averages in China as a whole (Zhang, 2016; Zeng and Zhao, 2019). The prevalence rate differences between males and females observed in this study align well with the data from other parts of China (Xue et al., 2018). Due to the acceleration of urbanization, improvement of living standards, and the heavy-flavored diet of the Northern population (i.e., due to excessive use of salt) (Fang et al., 2020), the Northern Chinese have a higher prevalence of hypertension compared to those in the South (Zhao et al., 2004). As the survey site is limited to the Northern region, the findings of the current study can only be generalized to Northern (Chinese) older adults.

Our study is among the first to explore the sex- and age-specific prevalence of MCI among adults aged 60 years and over in Northern China, and to analyze an extensive list of risk factors for MCI. Moreover, we used rigorous and standardized protocols and quality control procedures for data collection and adjusted for the age and sex structure of populations in the prevalence estimates to enable comparisons with other studies. However, this study has several limitations. Due to the cross-sectional nature of this study, the observed associations may be influenced by reverse causation, particularly for lifestyle-related factors. However, we performed several sensitivity analyses to evaluate the potential for reverse causation, and the results remained unchanged. Next, although numerous sociodemographic and health-related factors were retained in the final models, residual confounding may still exist. Furthermore, since we did not have data on estrogen levels, we were unable to explore potential mechanisms underlying the association between TG and cognitive function in older females. Therefore, future studies should measure estrogen to examine the reasons for the association between TG and cognitive function in older females more fully. Finally, since this study was a field survey, elderly adults with limited mobility were not included. Therefore, further household surveys are needed to yield more generalizable findings.

In conclusion, 10.7% of all adults aged 60 years and above were found to have MCI in this study. The prevalence of MCI was higher in females and older age groups. In addition, PA, BMI, and GS were inversely associated with MCI, whereas sleepiness, longer sleep duration, and hypertension tended to increase the prevalence of MCI. TG and BMI might have different associations with the presence of MCI at different sex and age stages, respectively. Our data highlight the need for mechanism studies to better understand differences in the associations between multiple influencing factors and MCI. Moreover, large prospective studies with detailed baseline data, follow-up of health-related factors, and cognitive impairment-related outcomes are required so that individual risk can be predicted and managed by calculating risk scores for cognitive impairment. Therefore, further prospective studies or clinical trials are required.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Tianjin Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JF contributed to the statistical analysis, interpretation of the data, and drafting of the manuscript. JF, QL, YD, CS, HL, and MJ contributed to the acquisition of the data. YD, YZ, FM, WL, HL, XZ, YC, ZS, GW, and GH contributed to the conception, design, and revision of the manuscript. JF, QL, HL, and MJ contributed to the assembly of the data. JF, QL, YD, GW, and GH contributed to the approval of the final version of the manuscript. All the authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Associations of Lifestyle Factors With Cognition in Community-Dwelling Adults Aged 50 and Older: A Longitudinal Cohort Study

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In the absence of an effective treatment to alter the progressive course of cognitive decline and dementia, identification of modifiable risk factors that could promote healthy cognitive aging has become a public health research priority. This study seeks to comprehensively determine the contemporaneous associations of a broad spectrum of time-varying modifiable lifestyle factors with age-related cognitive decline in a large population-based cohort of older adults. A total of 5,711 subjects aged 50 and older from the WHO Study on global AGEing and adult health (SAGE) in Shanghai were studied. Repeated measures of lifestyle factors and cognitive performance were conducted in 2009–2010 and 2014–2015. Linear random slope models were used to evaluate the contemporaneous associations between time-varying lifestyle factors and cognitive performance. Person-mean centering method was used to disaggregate the between- and within-person effects in the time-varying lifestyle factors in the random slope models. We found that higher vegetable and fruit consumption, as well as higher level of physical activity were positively associated with all cognitive domains. Body mass index (BMI) was negatively associated with all cognitive domains, whereas waist-to-hip ratio (WHR) was negatively associated with verbal fluency score only. Sedentary time was negatively associated with digit span score but positively associated with verbal fluency score. The between-person effects seem to be more dominant than within-person effects. Overall, our findings suggest better management of multiple lifestyle factors may protect against cognitive decline in later life. Higher vegetable and fruit consumption and physical activity are protective, whereas obesity is detrimental to cognitive decline in older adults. This study underpins the development of multi-domain lifestyle recommendations to promote healthy cognitive aging.

Keywords: aging, cognitive decline, lifestyle, vegetable, fruit, physical activity, obesity, sedentary time

INTRODUCTION

Aging is accompanied by cognitive decline which is evident from as early as age 45 (Singh-Manoux et al., 2012). Age-related cognitive decline manifests itself as some diminishment of many core cognitive abilities, including memory, attention, processing speed, and executive function (Park and Bischof, 2013). The different levels of cognitive decline range from mild cognitive impairment to dementia (Langa and Levine, 2014). Dementia is one of the major causes of disability and dependency among older people worldwide. Dementia currently affects approximately 50 million people worldwide, with the majority (63%) living in low- and middle-income countries (WHO, 2020b). The total number of people with dementia is projected to reach 82 million by 2030 and 152 million by 2050 (WHO, 2020b). The number of patients with dementia in China accounts for approximately a quarter of the entire population with dementia worldwide, with a prevalence of 5.30 and 12.7% for dementia and mild cognitive impairment, respectively, in people aged 60 years and older, and an incidence of 12.14 and 21.7 per 1,000 person-years for dementia and mild cognitive impairment, respectively, in people aged 65 years and older (Jia et al., 2020). Therefore, there is an urgent need to slow down cognitive decline and halt the progression to dementia.

Since no effective pharmacological treatment is currently available to cure dementia, a greater emphasis has been placed on identification of potentially modifiable risk factors that could delay or prevent cognitive decline. Findings from prior research have provided promising results linking several modifiable risk factors to cognitive impairment and dementia. It is estimated that around a third of Alzheimer's diseases cases worldwide might be attributable to seven potentially modifiable risk factors (diabetes, midlife hypertension, midlife obesity, physical inactivity, depression, smoking, and low educational attainment) (Norton et al., 2014). Over the past decades, emerging evidence suggests that a "poor" lifestyle such as obesity, physical inactivity, unhealthy diet, smoking, and heavy alcohol consumption are associated with faster cognitive decline in old age (Wajman et al., 2018). Such evidence highlights the potential of promoting healthy lifestyles to preserve cognition in normal aging.

To date, the majority of previous studies investigating the relationship between lifestyle factors and cognition have used cross-sectional designs (Arenaza-Urquijo et al., 2015). However, age-related cognitive decline is highly variable across individuals (Nyberg et al., 2012). The cross-sectional design cannot estimate the effects of lifestyle factors on cognitive decline over time at an intra-individual level. Evidence from longitudinal studies, cross-lagged panel analyses and intervention trials has accumulated during the past few decades. The cross-lagged panel model excels in taking temporal orders into account and thus sheds light on the temporal sequence and causal direction of the associations between lifestyle factors and cognition (Farina et al., 2016; Ihle et al., 2019; Zhao et al., 2020). Despite this, most studies focus on only one or two lifestyle factors (Lövdén et al., 2013; Kivipelto et al., 2018). Cognitive decline are multifactorial in nature (Kivipelto et al., 2018). Risk factors and protective factors often co-occur and interact across a person's lifespan, and the result of which determines the overall risk of cognitive decline (Kivipelto et al., 2018). Previous single-domain studies did not consider the possible interactive effects between different lifestyle factors, and have yielded mixed results (Kivipelto et al., 2018). Consequently, studies that target multi-domain lifestyle factors simultaneously are needed to provide overall and integrated recommendations for how to delay cognitive decline in the general population. Furthermore, while studying the effects of lifestyle behavior on health, most epidemiological studies assume relatively stable behavioral patterns. However, lifestyle behavior is subject to fluctuations over time, and these fluctuations are not taken into account when evaluating the effects of lifestyle behavior on future health status (Mulder et al., 1998). As a consequence, the effects of lifestyle behavior on health are based on one single measurement. Research into temporally parallel changes in lifestyle behavior and changes in cognition at an intra-individual level is rather scarce, although this kind of study will provide the best insight into the contemporaneous associations between lifestyle behavior and cognitive functioning. Lastly, the majority of the epidemiological studies on lifestyle factors and cognition have only been carried out in high-income countries (Kivipelto et al., 2018). There is little published data from low-income and middle-income countries, which are facing the greatest burden of dementia.

To fill the above mentioned gap, this study seeks to explore the contemporaneous associations between a broad spectrum of time-varying modifiable lifestyle factors and cognition, as well as potential interactive effects between different lifestyle factors, in normal aging in a large longitudinal population-based cohort of Chinese adults aged 50 and older.

MATERIALS AND METHODS

Study Population

Participants were drawn from a large ongoing population-based cohort study, the WHO Study on global AGEing and adult health (SAGE) in Shanghai. Detailed descriptions of SAGE have been previously described (Kowal et al., 2012). Briefly, SAGE is a longitudinal study collecting data on adults aged 50 and older from nationally representative samples in China, Ghana, India, Mexico, Russian Federation and South Africa. We enlarged the sample size of SAGE in Shanghai, China to obtain a sub-state representative sample using the same multistage clustered sampling method and survey instrument. At baseline (Wave 1) between 2009 and 2010, 8,629 community dwellers aged 50 and older were recruited from five districts of Shanghai, China. Of subjects recruited, 74 who used proxy, 418 who had a history of stroke, and 137 who did not complete cognitive tests at Wave 1 were excluded; leaving 8,000 participants eligible for this study. Among the eligible participants, 5,711 completed cognitive tests at Wave 2 between 2014 and 2015, and were included in this study. This study was approved by the Shanghai Center for Disease Control and Prevention Ethical Review Committee. All participants provided informed written consent.

Lifestyle Factors

Lifestyle factors included vegetable and fruit intake (100 g/day), physical activity, sedentary time (hours/day), body mass index (BMI) and waist-to-hip ratio (WHR). All lifestyle factors were assessed at both Wave 1 and Wave 2. Participants were asked to indicate the number of servings of fruits and vegetables they consume on a typical day. One serving of fruits and vegetables is equivalent to 50 g in Wave 1 questionnaire and 80 g in Wave 2 questionnaire. In order to unify the measuring units and make them more comparable to other studies, we then converted the self-reported number of servings of fruits and vegetables to 100 g/day as an indicator for general fruit and vegetable intake. Physical activity level was measured based on responses to questions drawn from the Global Physical Activity Questionnaire (WHO, 2020a). Three categories of physical activity (low, moderate, and high physical activity) were calculated from these questionnaire items, based on reported time spent on moderate or vigorous activities during work, recreational/leisure time, and transportation (International Physical Activity Questionnaire [IPAQ], 2005). Trained doctors measured participants' weight, height and waist circumference. BMI was calculated by dividing the body weight (in kilograms) by the height (in meters) squared (kg/m^2). WHR was calculated as waist measurement divided by hip measurement. Sedentary time (hours/day) was measured based on the time spend sitting or reclining on a typical day.

Cognitive Function

Cognitive function was assessed through the following cognitive tests: (1) immediate verbal recall to assess learning capacity and memory storage (Morris et al., 1989); (2) delayed verbal recall to assess memory retrieval (Morris et al., 1989); (3) digit span forward and backward to assess concentration, attention and immediate memory (The Psychological Corporation, 2002); and (4) verbal fluency test to assess ability to retrieve semantic memory information (Morris et al., 1989). For the immediate verbal recall test, interviewers read a list of 10 words aloud and asked participants to immediately recall as many words as possible in 1 min. This test was performed three times. The final score was the average of three tests. After 10 min, delayed verbal recall test was performed by asking participants to remember the list of words without interviewers repeating the list. Digit span test asked participants to repeat increasingly longer series of numbers; the total score was recorded as the longest digit span repeated without error. This test was then performed with participants repeating new sets of increasingly longer digit spans in reverse. Verbal fluency test consisted of naming as many animals as possible in 1 min; the total score was correct responses minus errors.

Covariates

Covariates included age, sex, education, smoking (non-smoker and smoker), alcohol drinking (non-drinker and drinker), and self-reported common chronic health conditions including arthritis, angina, diabetes, chronic lung disease, asthma, hypertension, depression, and cataract.

Statistical Analyses

All analyses were performed using Stata 16.0. Statistics were presented as mean (SD) or n (%) where appropriate. *T*-test for paired samples was used to test the differences between cognitive scores at Wave 1 and Wave 2. Since repeated measurements of cognitive performance and lifestyle factors are two-level hierarchical data, with measurement occasions at level 1 and individuals at level 2, we analyzed the associations of cognitive performance with lifestyle factors using linear random slope models with maximum-likelihood estimates. Specifically, regression models were fitted to each individual's longitudinal cognitive change, resulting in an average model for the sample (fixed effects) plus individual deviations from the average model (random effects). Subject ID was included as a random effect. Age as the time scale, common chronic health conditions and lifestyle factors were all time-varying variables. As for random effects we included subject ID to account for repeated data of the same individuals. Coefficient of determination (R^2) was presented to indicate the proportion of level 1 and level 2 variances explained by the covariates.

To disaggregate the between- and within-person effects in the time-varying lifestyle factors in random slope models, we used person-mean centering method which involves recalculating a single time varying lifestyle factor into separate between- and within-person predictor variables: at level 1, the person-mean centered time-varying lifestyle factor serves as a within-person predictor, representing the amount by which a person deviates from his or her own average at each time point (within-person effect); at level 2, the person means serve as a between-person predictor, representing each person's average, pooling over all time points (between-person effect) (Howard, 2015).

We first evaluated the associations of a single lifestyle factor with cognitive scores separately, and then included all lifestyle factors in the same model (full model). To test for potential interactions between different lifestyle factors, as well as interactions between lifestyle factor and demographic factors (age and sex), we then included interaction terms in the full model. Lastly, to allow for a time lag between lifestyle factors and cognitive functioning, we also examined the associations between baseline lifestyle factors and longitudinal cognitive scores.

RESULTS

Of 5,711 subjects who finished the cognitive tests at both Wave 1 and Wave 2 with a mean interval of 4.89 years, 2,640 (46.28%) were men and 3,064 (53.72%) were women. The subjects ranged in age from 50 to 95, with a mean age of 62.29 at baseline. As shown in **Table 1**, at baseline, our participants had a mean vegetable and fruit intake of 369 g/day, mean BMI of 24.71 kg/m^2, mean WHR of 0.89, mean sedentary time of 4.10 h/day, 59.66% had only a low level of physical activity. Compared with participants who dropped out from the second cognitive assessment (n = 2,289), those who completed both cognitive assessments (n = 5,711) were 2.12 years younger ($p < 0.01$), more likely to be smokers ($p < 0.01$) and drinkers ($p = 0.01$), more likely to have

TABLE 1 | Comparisons of baseline characteristics between participants completed both cognitive assessments and those who dropped out from the second cognitive assessment.

	Participants completed the first cognitive assessment		Participants completed both cognitive assessments		Participants dropped out from the second cognitive assessment		*P*[c]
	(*n* = 8,000)		(*n* = 5,711)		(*n* = 2,289)		
	Mean/*N*[a]	*SD*/%[b]	Mean/*N*[a]	*SD*/%[b]	Mean/*N*[a]	*SD*/%[b]	
Age	62.90	9.53	62.29	8.84	64.41	10.91	< 0.01
Sex							0.42
Men	3,691	46.57	2,640	46.28	1,051	47.30	
Women	4,235	53.43	3,064	53.72	1,171	52.70	
Education attainment							< 0.01
Lower than primary school	1,344	19.66	1,035	21.28	309	15.67	
Primary school	1,378	20.16	1,064	21.88	314	15.92	
Middle school	2,221	32.49	1,534	31.54	687	34.84	
High school	1,386	20.28	906	18.63	480	24.34	
College or higher	507	7.42	325	6.68	182	9.23	
Yearly income (¥)	20233.73	95276.82	19142.80	90898.20	22993.60	105520.00	0.12
Smoking	2,133	26.66	1,595	27.93	538	23.50	< 0.01
Alcohol drinking	1,560	80.48	1,155	20.25	405	17.72	0.01
Common chronic health conditions							
Hypertension	2,749	34.60	1,960	34.59	789	34.62	0.98
Arthritis	1,311	16.39	997	17.46	314	13.72	< 0.01
Cataract	813	10.43	544	9.80	269	11.98	< 0.01
Diabetes	654	8.19	442	7.75	212	9.27	0.03
Chronic lung disease	501	6.27	354	6.21	147	6.42	0.72
Angina	464	5.82	323	5.67	141	6.18	0.38
Asthma	176	2.21	115	2.03	61	2.68	0.08
Depression	37	0.46	28	0.49	9	0.39	0.56
Vegtable and fruit intake (*100g/day)	3.68	1.68	3.69	1.72	3.66	1.59	0.47
BMI	24.61	4.17	24.71	4.30	24.35	3.81	< 0.01
WHR	0.89	0.11	0.89	0.10	0.89	0.13	0.06
Physical activity level							0.56
Low level	4,801	60.01	3,407	59.66	1,394	60.90	
Moderate level	2,276	28.45	1,643	28.77	633	27.65	
High level	923	11.54	661	11.57	262	11.45	
Sedentary time (hours/day)	4.23	2.43	4.10	2.41	4.56	4.56	< 0.01
Immediate verbal recall score	5.83	1.66	5.87	1.64	5.75	1.70	< 0.01
Delayed verbal recall score	5.14	2.07	5.15	2.05	5.12	2.13	0.65
Digit span score	11.13	2.58	11.22	2.62	10.93	2.47	< 0.01
Verbal fluency score	12.53	5.36	12.34	5.25	12.99	5.60	< 0.01

[a]*Mean for continuous variables or number for categorical variables.* [b]*Standard deviation (SD) for continuous variables or percentage (%) for categorical variables.* [c]*Compared between participants who completed both cognitive assessments and those who dropped out from the second cognitive assessment.*

arthritis ($p < 0.01$) but less likely to have cataract ($p < 0.01$) and diabetes ($p = 0.03$), had a lower education attainment ($p < 0.01$), a higher BMI ($p < 0.01$), shorter daily sedentary time ($p < 0.01$), a higher immediate verbal recall score ($p < 0.01$) and digit span score ($p < 0.01$), and a lower verbal fluency score ($p < 0.01$).

Table 2 compares the cognitive scores at Wave 1 and Wave 2. The mean scores in all cognitive domains significantly declined from Wave 1 to Wave 2. The mean score of immediate verbal recall decreased from 5.87 to 5.35 ($p < 0.01$), delayed verbal recall from 5.15 to 4.98 ($p < 0.01$), digit span from 11.22 to 10.00 ($p < 0.01$), and verbal fluency from 12.34 to 10.99 ($p < 0.01$).

Table 3 shows the contemporaneous associations between lifestyle factors and cognitive scores from Wave 1 to Wave 2, with separate between- and within-person effects. Vegetable and fruit intake was positively associated with scores in all cognitive domains ($p < 0.01$). Per 100 g/day increase in vegetable and fruit intake was associated with an increase of 0.07 in immediate verbal recall score, 0.08 in delayed verbal recall score, 0.07 in digit span score, and 0.39 in verbal fluency score. The between-person

TABLE 2 | Comparisons of cognitive scores at Wave 1 and Wave 2.

Cognitive domain	Wave 1		Wave 2		Difference		
	N	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*p*
Immediate verbal recall	5,705	5.87 (1.64)	5,590	5.35 (1.89)	5,584	−0.51(2.30)	<0.01
Delayed verbal recall	5,626	5.15 (2.05)	5,557	4.98 (2.15)	5,473	−0.17(2.72)	<0.01
Digit span	5,633	11.22(2.62)	5,676	10.00(3.35)	5,599	−1.19(3.81)	<0.01
Verbal fluency	5,701	12.34(5.25)	5,168	10.99(5.04)	5,158	−1.34(6.22)	<0.01

effects were significant for all cognitive domains ($p < 0.01$), while the within-person effects were only significant for immediate verbal recall ($p = 0.02$) and verbal fluency ($p < 0.01$). For different persons at the same age, per 100 g/day increase in vegetable and fruit intake was associated with an increase of 0.10 in immediate verbal recall score, 0.14 in delayed verbal recall score, 0.09 in digit span score, and 0.37 in verbal fluency score (cross-sectional difference). For the same person from Wave 1 to Wave 2, per 100 g/day 5 year increase in vegetable and fruit intake was associated with a 5 year increase of 0.04 in immediate verbal recall score, and 0.40 in verbal fluency score (longitudinal change). Likewise, physical activity level was positively associated with scores in all cognitive domains ($p < 0.01$). Compared with subjects who had a low level of physical activity, those who had a moderate level of physical activity scored 0.17, 0.16, 0.16, and 0.99 higher in immediate verbal recall, delayed verbal recall, digit span, and verbal fluency, respectively. The between-person effects were significant for all cognitive domains ($p < 0.01$), while the within-person effects were only significant for immediate verbal recall ($p < 0.01$), delayed verbal recall ($p < 0.01$), and verbal fluency ($p < 0.01$). In contrast, BMI was negatively associated with scores in all cognitive domains ($p < 0.01$). One unit (kg/m^2) increase in BMI was associated with a decrease of 0.02 in immediate verbal recall score, 0.03 in delayed verbal recall score, 0.03 in digit span score, and 0.05 in verbal fluency score. The between-person effects were significant for all cognitive domains ($p < 0.01$), while the within-person effects were only significant for delayed verbal recall ($p < 0.01$). WHR was negatively associated with verbal fluency score only ($p = 0.01$), and only its within-person effect was significant ($p < 0.01$). Sedentary time was negatively associated with digit span score ($\beta = -0.03$, $p < 0.01$), with a significant within-person effect ($p < 0.01$), but positively associated with verbal fluency score ($\beta = 0.15$, $p < 0.01$), with significant between- and within-person effects ($p < 0.01$). When all lifestyle factors were included in the same model (full model), all aforementioned associations remained unchanged, except the insignificant association of WHR with digit span score became significant ($p = 0.045$).

Table 4 presents the interactions between different lifestyle factors, as well as interactions between lifestyle factor and demographic factors (age and sex). Significant interactions were observed between age and all lifestyle factors: The effects of one unit increase in vegetable and fruit intake (100 g/day), BMI, and physical activity level on digit span score increased by 0.005 ($p = 0.046$), 0.002 ($p = 0.024$), and 0.014 ($p = 0.013$), respectively, when age increased by one unit; the effects of one unit increase in BMI and sedentary time (1 h/day) on delayed verbal recall score changed by 0.001 ($p = 0.041$) and −0.003 ($p = 0.009$), when age increased by one unit. None of the interactions between sex and lifestyle factors was significant, indicating the associations of lifestyle factors with cognitive scores did not differ by sex. Among the interactions between every two lifestyle factors, only vegetable and fruit intake had significant interactions with all other lifestyle factors: For every unit increase in vegetable and fruit intake (100 g/day), the effect of BMI on delayed verbal recall score decreased by 0.009 ($p = 0.018$); the effect of WHR on immediate verbal recall score decreased by 0.337 ($p = 0.020$); the effect of physical activity level on digit span score increased by 0.054 ($p = 0.045$); and the effect of sedentary time on immediate verbal recall score and digit span score increased by 0.010 ($p = 0.020$) and 0.021 ($p = 0.005$), respectively.

To allow for a time lag between lifestyle factors and cognitive changes, we also examined the associations between baseline lifestyle factors and longitudinal cognitive scores (**Supplementary Table 1**), as well as the interactions between different baseline lifestyle factors and the interactions between baseline lifestyle factors and demographic factors (age and sex) (**Supplementary Table 2**). These results differ from the contemporaneous associations in **Tables 3**, **4**, which supports our assumption that the fluctuations in lifestyle behavior over time affect the predictive value of lifestyle behavior for future cognitive performance.

DISCUSSION

This large-scale prospective cohort study has been one of the first attempts to examine the contemporaneous associations of a broad spectrum of time-varying modifiable lifestyle factors with cognitive performance in older Chinese adults. Overall, our study suggests that better management of multiple lifestyle factors may protect against cognitive decline in later life. Specifically, we have found that higher vegetable and fruit consumption and a higher level of physical activity were positively associated with all cognitive domains, whereas BMI was negatively associated with all cognitive domains. Sedentary time was negatively associated with digit span score but positively associated with verbal fluency score. The between-person effects seem to be more dominant than within-person effects.

Our results seem to be consistent with previous research which has shown that the adherence to overall healthier lifestyles are associated with better cognitive function in adulthood (Lövdén

TABLE 3 | Contemporaneous associations between lifestyle factors and cognitive scores (between- and within-person effects).

	Immediate verbal recall						Delayed verbal recall						Digit Span						Verbal fluency					
	Estimate	*SE*	p^a	R^2	ICC[b]	*p* (full model)[c]	Estimate	*SE*	p^a	R^2	ICC[b]	*p* (full model)[c]	Estimate	*SE*	p^a	R^2	ICC[b]	*p* (full model)[c]	Estimate	*SE*	p^a	R^2	ICC[b]	*p* (full model)[c]
Vegetable and fruit intake (*100g/day)	0.07	0.01	< 0.01		0.12	< 0.01	0.08	0.01	< 0.01		0.04	< 0.01	0.07	0.02	< 0.01		0.07	< 0.01	0.39	0.03	< 0.01		0.22	< 0.01
Between-person effect	0.1	0.02	< 0.01	0.13			0.14	0.02	< 0.01	0.13			0.09	0.03	< 0.01	0.14			0.37	0.05	< 0.01	0.15		
Within-person effect	0.04	0.02	0.02	0.09			0.02	0.02	0.32	0.09			0.05	0.03	0.07	0.1			0.4	0.04	< 0.01	0.12		
BMI	−0.02	0	< 0.01		0.21	< 0.01	−0.03	0.01	< 0.01		0.04	< 0.01	−0.03	0.01	< 0.01		0.17	< 0.01	−0.05	0.01	< 0.01		0.23	< 0.01
Between-person effect	−0.02	0.01	< 0.01	0.13			−0.02	0.01	< 0.01	0.13			−0.03	0.01	< 0.01	0.13			−0.06	0.02	< 0.01	0.15		
Within-person effect	−0.01	0.01	0.15	0.09			−0.05	0.01	< 0.01	0.09			−0.03	0.01	0.07	0.1			0	0.03	0.9	0.11		
WHR	−0.09	0.2	0.65		0.21	0.77	−0.12	0.24	0.62		0.03	0.53	0.39	0.33	0.25		0.07	0.045	−1.93	0.6	< 0.01		0.24	0.02
Between-person effect	−0.12	0.27	0.67	0.09			−0.15	0.32	0.65	0.13			−0.12	0.46	0.8	0.13			−0.24	0.87	0.79	0.15		
Within-person effect	−0.06	0.3	0.84	0.12			−0.08	0.35	0.82	0.09			0.93	0.49	0.06	0.1			−3.65	0.87	< 0.01	0.11		
Physical activity level	0.17	0.02	< 0.01		0.19	< 0.01	0.16	0.03	< 0.01		0.02	< 0.01	0.16	0.04	< 0.01		0.09	< 0.01	0.99	0.07	< 0.01		0.19	< 0.01
Between-person effect	0.16	0.03	< 0.01	0.13			0.15	0.04	< 0.01	0.13			0.28	0.06	< 0.01	0.14			1.2	0.1	< 0.01	0.17		
Within-person effect	0.18	0.04	< 0.01	0.09			0.16	0.04	< 0.01	0.09			0.03	0.06	0.6	0.1			0.8	0.1	< 0.01	0.13		
Sedentary time (hours/day)	0.01	0.01	0.19		0.18	0.2	0.01	0.01	0.18		0.01	0.18	−0.03	0.01	< 0.01		0.11	< 0.01	0.15	0.02	< 0.01		0.17	< 0.01
Between-person effect	0.01	0.01	0.16	0.12			0.02	0.01	0.1	0.12			0	0.02	0.91	0.13			0.18	0.03	< 0.01	0.15		
Within-person effect	0	0.01	0.66	0.09			0	0.01	0.85	0.09			−0.07	0.02	< 0.01	0.1			0.12	0.03	< 0.01	0.12		

[a]*Adjusted for age, sex, education, smoking, drinking, and self-reported common chronic health conditions including arthritis, angina, diabetes, chronic lung disease, asthma, hypertension, depression and cataract.* [b]*Conditional intraclass correlation.* [c]*Additionally adjusted for all other lifestyle factors of this study.*

TABLE 4 | Contemporaneous associations between lifestyle factors and cognitive scores including interactions.

	Immediate verbal recall			Delayed verbal recall			Digit span			Verbal fluency		
	Estimate	***SE***	***p***	**Estimate**	***SE***	***p***	**Estimate**	***SE***	***p***	**Estimate**	***SE***	***p***
Vegetable and fruit intake (*100g/day)	0.196	0.164	0.234	0.369	0.197	0.061	−0.115	0.275	0.677	0.756	0.488	0.121
Vegetable and fruit intake*age	0.002	0.001	0.253	0.001	0.002	0.530	0.005	0.002	0.046	0.004	0.004	0.333
Vegetable and fruit intake*sex	0.033	0.023	0.195	0.005	0.027	0.903	0.014	0.038	0.739	0.002	0.068	0.924
BMI	−0.137	0.066	0.030	−0.171	0.079	0.030	−0.166	0.111	0.140	−0.199	0.197	0.304
BMI*age	0.001	0.001	0.142	0.001	0.001	0.041	0.002	0.001	0.024	0.003	0.002	0.103
BMI*sex	0.016	0.010	0.109	0.011	0.012	0.338	−0.019	0.016	0.266	0.014	0.029	0.631
WHR	2.561	2.249	0.255	2.982	2.690	0.267	7.088	3.768	0.059	10.490	6.653	0.115
WHR*age	−0.018	0.027	0.504	−0.039	0.033	0.238	−0.084	0.046	0.070	−0.114	0.081	0.159
WHR*sex	−0.580	0.469	0.215	−0.792	0.562	0.159	−0.357	0.784	0.649	−2.396	1.396	0.086
Physical activity level	0.145	0.400	0.717	0.522	0.480	0.277	−1.232	0.670	0.066	2.261	1.189	0.057
Physical activity level*age	0.004	0.003	0.268	−0.001	0.004	0.728	0.014	0.006	0.013	−0.012	0.010	0.237
Physical activity level*sex	0.010	0.054	0.843	0.038	0.064	0.552	−0.025	0.090	0.787	−0.208	0.160	0.202
Sedentary time (hours/day)	0.142	0.107	0.205	0.118	0.128	0.356	0.227	0.180	0.207	0.287	0.317	0.365
Sedentary time*age	−0.001	0.001	0.249	−0.003	0.001	0.009	−0.002	0.001	0.075	−0.003	0.002	0.238
Sedentary time*sex	−0.013	0.015	0.414	0.000	0.017	0.963	−0.030	0.024	0.215	−0.085	0.044	0.050
Vegetable and fruit intake*BMI	−0.002	0.003	0.549	−0.009	0.004	0.018	0.002	0.005	0.730	−0.005	0.009	0.583
Vegetable and fruit intake*WHR	−0.337	0.145	0.020	−0.232	0.173	0.180	−0.386	0.242	0.110	−0.632	0.428	0.139
Vegetable and fruit intake*physical activity level	0.016	0.016	0.336	0.021	0.019	0.279	0.054	0.027	0.045	−0.021	0.047	0.627
Vegetable and fruit intake*sedentary time	0.010	0.004	0.020	0.005	0.005	0.388	0.021	0.007	0.005	0.010	0.013	0.411
BMI*WHR	0.064	0.050	0.229	0.088	0.060	0.158	0.028	0.085	0.745	0.005	0.150	0.993
BMI*physical activity level	−0.002	0.007	0.852	−0.004	0.009	0.713	−0.001	0.012	0.930	0.011	0.021	0.571
BMI*sedentary time	0.001	0.002	0.531	0.000	0.002	0.966	−0.001	0.003	0.770	−0.010	0.006	0.087
WHR*physical activity level	−0.292	0.339	0.388	−0.455	0.407	0.254	0.312	0.567	0.578	−0.602	1.005	0.549
WHR*sedentary time	−0.128	0.099	0.194	0.016	0.118	0.881	−0.149	0.166	0.355	0.438	0.293	0.135
Physical activity level*sedentary time	0.000	0.011	0.990	0.014	0.013	0.259	0.019	0.018	0.287	−0.015	0.032	0.657

Adjusted for age, sex, education, smoking, drinking, and self-reported common chronic health conditions including arthritis, angina, diabetes, chronic lung disease, asthma, hypertension, depression and cataract.

et al., 2013; Klimova et al., 2017; Kivipelto et al., 2018; Bott et al., 2019; Mintzer et al., 2019). The mechanisms that underlie the relationship between lifestyle factors and cognition are not yet fully understood. Several hypotheses have been proposed that highlight the involvement of vascular, inflammatory, oxidative stress, neurotoxic and psychosocial processes (Kivipelto et al., 2018). Much more extensive research is available in the area of diet and physical activity compared to other lifestyle factors. Overall, fruits and vegetables were the most common dietary elements associated with better cognitive function. This study confirms that higher vegetable and fruit consumption and physical activity are protective against cognitive decline in older adults. Vegetables and fruits are rich in antioxidant vitamins and nutrients, compounds that are considered important for the protection against oxidative stress and inflammation, which, in turn, have been shown to play a role in the early pathophysiology of cognitive decline (Hajjar et al., 2018; Gehlich et al., 2019). Moreover, vegetables and fruits might affects the composition of the gut microbiota and stimulate a positive modulation of the gut–brain axis, which might be another mechanistic pathway to impact cognitive health (Pistollato et al., 2016). A previous prospective study of 3,718 elderly participants reported that the decrease in cognitive decline over 6 years of follow-up for people who consumed greater than two vegetable servings per day was equivalent to about 5 years of younger age on cognitive testing (Morris et al., 2006). Similarly, strong observational data have identified physical activity as a potent lifestyle factor that plays a critical role in alleviating age-related cognitive decline across the life span (Prakash et al., 2015; Engeroff et al., 2018). It is hypothesized that the neural and vascular adaptations to physical activity improve cognition through promotion of neurogenesis, angiogenesis, synaptic plasticity, decreased proinflammatory processes and reduced cellular damage due to oxidative stress (Northey et al., 2018). A meta-analysis of cohort studies concluded that as compared with adults not engaged in physical activity, those with a high level of physical activity showed 38% less decline in cognitive performance during a 1–12 years of follow-up, and even those with a low-to-moderate level of physical activity also showed 35% less decline (Sofi et al., 2011). A more recent meta-analysis examining the effects of exercise interventions on cognitive outcomes in adults aged 50 and older reported an overall small-to-moderate, but significant, effect size for all outcomes of cognition evaluated (i.e., attention, executive function, memory, and working memory), which is in agreement with our findings (Northey et al., 2018).

On the other hand, the existing literature on the relationship between obesity and cognition has yielded contradictory and age dependent findings. While obesity in midlife appears to be detrimental to cognitive decline, obesity in the old (over 65 years) has been reported to be detrimental, neutral or even protective, highlighting an "obesity paradox" (Sellbom and Gunstad, 2012; Bischof and Park, 2015; Monda et al., 2017). These paradoxical findings may be explained by a survival effect in elderly samples. Namely, some middle-aged obese adults experience mortality, leaving a healthier sample of obese adults in old age (Smith et al., 2011; Bischof and Park, 2015). Furthermore, some researchers have argued that BMI, which is the most widely used indicator of obesity in studies with respect to cognitive decline, is not a good measure of body composition in the old (Bischof and Park, 2015). Lean body mass decreases, while adipose tissue increases without weight gain during aging (Monda et al., 2017). This distribution of lean and fat tissue masses may not be captured by BMI when evaluating adiposity in the elderly (Monda et al., 2017). Central adiposity (i.e., waist circumference or waist-to-hip ratio), on the other hand, has been proposed as a better adiposity marker in old age (Bischof and Park, 2015; Monda et al., 2017). On the question of the "obesity paradox," we further investigated the relation of obesity and cognitive performance stratified by baseline age ($\leq$65 and $>$65) (**Supplementary Table 3**). Our stratified analysis showed that the negative associations of BMI with all cognitive domains were only significantly in people up to 65 years old ($p < 0.01$), but not in people older than 65 ($p > 0.05$). The positive association of WHR with digit span was only significant in people up to 65 years old in the full model ($p = 0.01$), while the negative association of WHR with verbal fluency was only significant in people older than 65 ($p = 0.01$). The results of our stratified analysis seems to reflect the aforementioned "obesity paradox" phenomenon and differential findings of BMI and WHR with respect to cognitive decline. Overall there is currently not enough evidence to conclude a reliable association between obesity and cognitive decline in older adults. More research is needed to understand the complex nature of the effects of obesity on cognition.

Our study found that the between-person associations were more dominant than the within-person associations for some lifestyle factors, particularly for BMI, which might be attributed to the relatively short follow-up period and the extent of within-person changes in lifestyle factors. First, our study accessed the within-subject effects over 5 years, however, the within-subject effects might need a longer follow-up period to manifest. Second, small within-person changes in lifestyle factors might not result in significant effects on cognitive functioning. The extent of within-person changes varied widely among different lifestyle factors in our participants. Within a 5-year follow-up period, only 30.5% of the participants changed BMI group, 40.1% changed WHR group, 51.2% changed physical activity level, 70.0% had more than 20% change in vegetable and fruit intake, and 80.9% had more than 20% change in sedentary time. The stability in BMI seems to explain the insignificant within-person associations for all cognitive domains. More significant within-person associations were observed for other lifestyle factors with greater within-person changes, such as WHR, physical activity level, vegetable and fruit intake, and sedentary time. Nevertheless, people should engage in a healthy lifestyle, so that it could have an impact on cognitive function in later life.

Our study found many significant interactions between different lifestyle factors, suggesting potential interactive effects among lifestyle factors on cognition. The etiology of cognitive decline and dementia is multifactorial and has many concurrent modifiable risk factors and protective factors (Kivipelto et al., 2018). To date, lifestyle intervention studies that have aimed to prevent cognitive decline and dementia have mainly been

single-domain trials, in which only single behavior was targeted (Lövdén et al., 2013; Kivipelto et al., 2018). Such single-domain approach did not consider the possible interactive effects between different lifestyle factors, and is unable to provide overall and integrated recommendations for how to slow down cognitive decline and prevent dementia in the general population. Consequently, studies targeting many lifestyle factors simultaneously may help understand the combined effects of lifestyle factors on cognitive decline, and offer the best approach to an optimal preventive effect. Evidence from the first large multi-domain lifestyle intervention trial (FINGER) has provided some support for the conceptual premise that multi-domain lifestyle intervention is an effective strategy (Ngandu et al., 2015). However, two other large multi-domain lifestyle intervention trials (MAPT and PreDIVA) have found negative results (Moll van Charante et al., 2016; Andrieu et al., 2017). In addition to these three large multi-domain trials, several small-scale trials have yielded conflicting findings too. Owing to the limited sample size and duration of these trials, the efficacy of multi-domain lifestyle intervention for cognitive decline remains inconclusive. Further research exploring the combined effects of a broad range of lifestyle factors on cognitive decline with a long follow-up period is warranted.

Strengths of this study include the longitudinal cohort design, a large population-based sample, and a long period of follow-up. Further strength includes repeated measures of synchronous lifestyle factors and cognitive performances and the evaluation of contemporaneous associations between them, which is rarely available in prior research. Most notably, this study examined a broad range of lifestyle factors on cognitive decline simultaneously, and therefore could provide overall and integrated recommendations on prevention against cognitive decline. A limitation of this study is the potential attrition bias due to differential loss to follow-up with respect to the lifestyle variables and cognitive outcomes. Another limitation is that the cognitive measures used in this study do not have immediate clinical relevance in terms of risk for mild cognitive impairment (MCI) and dementia. Future research that uses MCI as a measure of cognitive functioning would have more important implications at a population level. Finally, our sample is only representative of the older adults in Shanghai, not all of China.

In summary, the present study has been one of the first attempts to comprehensively determine the contemporaneous associations of a broad spectrum of time-varying modifiable lifestyle factors with age-related cognitive decline in older adults. Our findings suggest that better management of multiple lifestyle factors may protect against cognitive decline in later life. Higher vegetable and fruit consumption and physical activity are protective, whereas obesity is detrimental to cognitive decline in older adults. This study underpins the development of multi-domain lifestyle recommendations to promote healthy cognitive aging. As lifestyle behavior is a self-regulating and relatively easy target to prevent cognitive decline, the public health relevance of such a non-pharmacological approach is an important consideration. In the context of the fast growing aging population and dementia patients, even modest positive effects on cognitive function related to greater adherence to an overall healthy lifestyle could yield significant public health benefits if they can be cost-effectively delivered on a large scale. Especially in low- and middle-income countries with limited health-care resources, lifestyle intervention might represent one cost-effective general prevention strategy. More longitudinal randomized controlled trials targeting multi-domain lifestyle intervention are needed to determine the effectiveness of multi-domain lifestyle intervention and inform future lifestyle recommendations for older adults to prevent cognitive decline.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Shanghai Center for Disease Control and Prevention Ethical Review Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZH contributed to the design of the study, data collection and analysis, and preparation of the manuscript. YG, YR, SS, YS, and FW contributed to the design of the study, data collection, and preparation of the manuscript. TL, JY, JL, LH, and SW contributed to the data collection and processing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Aging Neuroscience

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Perceptual Priming Can Increase or Decrease With Aging

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A decline in declarative or explicit memory has been extensively characterized in cognitive aging and is a hallmark of cognitive impairments. However, whether and how implicit perceptual memory varies with aging or cognitive impairment is unclear. Here, we compared implicit perceptual memory and explicit memory measures in three groups of participants: (1) 59 healthy young volunteers (20–30 years); (2) 269 healthy old volunteers (50–90 years) and (3) 21 patients with mild cognitive impairment, i.e., MCI (50–90 years). To measure explicit memory, participants were tested on standard recognition and recall tasks. To measure implicit perceptual memory, we used a classic perceptual priming paradigm. Participants had to report the shape of a visual search pop-out target whose color or position was varied randomly across trials. Perceptual priming was measured as the speedup in response time for targets that repeated in color or position. Our main findings are as follows: (1) Explicit memory was weaker in old compared to young participants, and in MCI patients compared to age- and education-matched controls; (2) Surprisingly, perceptual priming did not always decline with age: color priming was smaller in older participants but position priming was larger; (3) Position priming was less frequent in the MCI group compared to matched controls; (4) Perceptual priming and explicit memory were uncorrelated across participants. Thus, perceptual priming can increase or decrease with age or cognitive impairment, but these changes do not covary with explicit memory.

Keywords: perception, priming, implicit memory, perceptual priming, visual search, aging, mild cognitive impairment

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INTRODUCTION

Memory has broadly been classified into explicit and implicit memory (Squire, 1992; Schacter et al., 1993; Gazzaniga et al., 2014). Explicit memory is consciously accessible and declarative; it is measured by how well participants can recall items that were previously studied (Strauss et al., 2006). By contrast, implicit memory is unconscious and non-declarative; it is measured by the facilitation in the response to previously experienced items (Schacter et al., 1993; Fleischman et al., 2005; Spataro et al., 2016). Since a decline in explicit memory is a hallmark of both aging and cognitive disorders, the question of whether implicit memory is also affected has been extensively investigated (Mitchell and Bruss, 2003; Fleischman, 2007; Berry et al., 2008; Ward et al., 2013). In most studies, implicit memory is measured as an increased probability of producing a studied item in an unrelated task, or by the facilitated recognition of a fragmented picture after it was previously viewed. The results are mixed: explicit memory always shows a clear decline with age and cognitive

impairments, but implicit memory declines in some cases (Perri et al., 2007; Soldan et al., 2009; Ballesteros et al., 2013; Gordon et al., 2013; Boccia et al., 2014) but not others (Fleischman et al., 2005; LaVoie and Faulkner, 2008). Nonetheless it has been proposed that an implicit memory deficit could be an early sign for the onset of dementia (Fleischman, 2007).

Despite these insights, the commonly used implicit memory tests have several problems. First, participants may use explicit memory during the study phase. This "explicit contamination" can be mitigated but is extremely tricky to fully rule out (Fleischman, 2007). Second, these tests assume a minimum proficiency in verbal and object naming which may vary widely especially in diverse populations with varying degrees of multilingualism and literacy.

One potential solution to these issues is to develop tasks that are culture-free with no study phase. Recent studies have addressed this issue by measuring the facilitation in categorical responses upon repeated viewing of objects, and have shown that this priming is weaker for older participants but only for unfamiliar objects (Soldan et al., 2009; Gordon et al., 2013). While these tasks require processing complex object properties, they have the advantage that they enable the comparison of implicit and explicit memory for the same items.

Here, we devised an implicit memory paradigm based on a classic perceptual priming effect, known as priming of pop-out (Maljkovic and Nakayama, 1994, 1996). In this paradigm, participants are faster to respond to the shape of a pop-out target when its color or position is repeated. This task has several advantages over implicit verbal memory measures. First, the task is easy to comprehend and assumes no prior knowledge of objects or words, making it suitable for use on diverse populations. Second, the task does not involve a study phase, thereby mitigating any explicit contamination. Third, since participants have to report the target shape while its color and position are manipulated independently, the speedup in their responses due to making repeated responses (i.e., motor priming) can be decoupled from any effects of repeated target color or position. However, this task suffers from having very little variation in item shape, with the result that explicit and implicit memory cannot be compared for the same items (Schacter et al., 1993; Constantinidou and Baker, 2002). Although priming of pop-out is a well-known paradigm, how this effect varies across age groups or across cognitive disorders and whether it covaries with explicit memory has never been investigated previously. Our goal therefore was to characterize how this particular form of implicit perceptual memory varies across age and cognitive impairments and assess whether it covaries with explicit memory measures.

MATERIALS AND METHODS

All participants had normal or corrected-to-normal vision and gave written informed consent to an experimental protocol approved by the Institutional Human Ethics Committee of the Indian Institute of Science, Bangalore. All experiments were conducted in accordance with the relevant guidelines and regulations. All participants were compensated monetarily for their participation.

Participants

Young volunteers were all students from the Indian Institute of Science campus. Older volunteers were all from an urban, literate background and recruited through extensive community engagement, and were all participants of an ongoing Tata Longitudinal Study of Aging (TLSA). Older volunteers underwent standard clinical and neuropsychological evaluations, based on which they were labeled as healthy or MCI (McKhann et al., 2011). The experimenters performing this study were blind to these labels during data collection and were given the label only afterward for the purposes of analysis. The older volunteers were larger in number since they were part of an ongoing study, whereas the younger volunteers were recruited for the purposes of the age comparisons in this study.

In all, we analyzed data from 59 young participants (25 ± 3 years, 29 female; years of education: 19 ± 3 years), 269 old participants (67 ± 8 years, 119 female; years of education: 19 ± 3 years), and 21 MCI patients (72 ± 10 years, 4 female; years of education: 15 ± 5 years). From these older participants, we selected a subset of participants whose age was similar to the MCI patients, and with similar number of years of education. This age- and education-matched control group (hereafter referred to as matched controls) comprised 92 participants (70 ± 10 years, 45 female; years of education: 16 ± 2 years).

MCI Diagnosis

The Clinical Dementia rating (CDR) scale (Hughes et al., 1982) was administered by trained research staff and a diagnosis was given based upon the scores to all the TLSA participants. CDR is administered by interviewing the volunteer and their primary caregiver (typically a close relative), which took about 20–30 min. A score of 0 corresponded to a healthy volunteer and 0.5 to MCI. MCI patients identified among the TLSA participants were broadly classified as amnestic or non-amnestic type. All the MCI patients ($n = 21$) in this study were of the amnestic type.

Global Cognitive Scores

To validate the MCI Diagnosis obtained from the CDR scale, we also compared the global cognitive score ACE III (Hsieh et al., 2013), measured from the older volunteers as part of the Tata Longitudinal Study of Aging. As expected, ACE-III scores were significantly higher for matched controls compared to MCI patients (ACE-III score, mean $\pm$ std: 94 ± 4 for matched controls, 87 ± 7 for MCI patients, cohen's d = 1.22, $p < 0.00005$, rank-sum test).

Procedure

All tasks were administered through HTML5/Javascript scripts run on an internet browser on a desktop computer (24-inch, width x height: 53.3 × 30.0 cm, 1920 × 1080 pixels) or laptop computer (15.4-inch, width × height: 33.0 × 20.6 cm, 2880 × 1800 pixels). All displayed items were scaled using the monitor size and viewing distance so that they subtended

the same visual angle. The browser-based setup was validated by comparing it with visual search tasks written in Matlab and Psychtoolbox. Each participant performed a perceptual priming task, an object recognition task, a word recall and word recognition task in that order, as detailed below.

Perceptual Priming Task

Each trial started with a gray fixation square (0.4° × 0.4°) displayed for 750 ms at the center of the screen, followed by a hexagonal search array (with items placed 6° from the center) containing one oddball colored item among five other distractors (**Figure 1A**). Each target or distractor shape was a diamond measuring 2° along the longer dimension with a 1° vertical cut on the left or right side. The array comprised a red target among green distractors or vice-versa, with the target chosen to appear randomly either at the leftmost or the rightmost location. Participants were instructed to indicate whether the oddball target diamond was cut on the right or left side by pressing the corresponding arrow key on a keyboard, and were asked to respond as quickly and accurately as possible. The search array was displayed for 10 s, or until the participant made a valid response, whichever was shorter. Target color and position were counterbalanced (i.e., equal numbers of red/green x left/right trials) and presented in random order. Error trials and trials with no response were repeated later after a random number of other trials. Participants performed a total of 200 correct trials.

Priming strength was calculated as the percent decrease in response time on a trial preceded by a target of the same color/position relative to the response time on trials where the target was of a different color/position. Thus,

$$priming\ strength\ =\ 100^{*}(DRT\ -\ SRT)/DRT$$

where, *DRT* is the mean reaction time of trials preceded by a different color/position compared to the current trial and *SRT* is the mean reaction time of trials preceded by the same color/position compared to the current trial. In the analyses reported here, we calculated priming strength using only consecutive trials with correct responses, but we obtained qualitatively similar results upon using all trials regardless of error status. We also obtained qualitatively similar results on using SRT in the denominator instead of DRT.

Since the trials were presented in random order, the different groups of participants might have slightly different numbers of trials that entered the SRT and DRT calculations. However, this was not the case: all groups had similar numbers of trials (mean ± sd of number of trials for SRT and DRT: 94 ± 9 and 90 ± 10 for young; 95 ± 8 and 92 ± 8 for old; 92 ± 10 and 92 ± 9 for MCI patients; 96 ± 8 and 91 ± 8 for matched controls.

Object Recognition Task

Each participant was asked to study a total of 20 objects (measuring 3.5° along the longer dimension) arranged in a 4 × 5 grid for 2 min, and were informed that they would be tested on these objects afterward. These were pictures of common objects such as animals, household objects, vehicles, etc., (**Figure 1B**). After the study phase, 40 pictures were presented, one at a time, prompting the participant to press 'y' or 'n' key to indicate whether (s)he saw the object during the study phase or not, with no time restriction on each response. Half of these were old (i.e., from the study phase) and the remaining half were new. Each new image was from the same basic-level category as the corresponding old image. The participants were instructed that they do not have to name any picture or for that matter give any descriptive account. Object recognition memory performance was characterized for each participant by calculating the total percentage correct across old and new items.

Word Recall and Recognition Tasks

Each participant performed a word recall task and a recognition task (McMinn et al., 1988), always in this order (**Figure 1C**). The word recall task started with a study phase in which 15 words (e.g., color, garden, coffee, house, etc.) were presented on the screen in a predefined sequence, with each word shown for 3 s. This was followed by a recall phase in which the participants were asked to recall as many words as they could from the study phase, and the experimenter typed in the words. Participants were free to recall words in any order and take any amount of time. The task was stopped once the participant declared that they could not remember any more words. Word recall performance was calculated for each participant as the fraction of words correctly recalled out of the full list.

During the recognition task, one word was presented at a time and the participant was asked to report with a key press, if the word was presented in the recall block ('y' for yes and 'n' for no). A total of 30 words were presented, 15 of which belonged to the recall block and 15 were new. The new words were drawn from similar categories as the study words (e.g., crayon, tree, home, etc.). Words did not overlap in content with the objects used in the object recognition task. Word recognition performance was calculated for each participant as the fraction of old and new words that elicited a correct response. Because our cohort was mostly urban and literate, all participants were assumed to be familiar with the words being shown, so it is unlikely that word novelty would affect recall or recognition. We also did not find any systematic relation between the number of years of education and the explicit memory measures.

Statistical Testing

Since accuracy and response times are frequently non-normal, we used non-parametric tests to compare participant groups (Wilcoxon signed-rank test for paired comparisons and Wilcoxon rank-sum test for unpaired comparisons). We used ANOVA for multifactorial comparisons since there are no non-parametric analogs. Both these tests take into account the unequal sample sizes across groups. Using parametric or nonparametric tests yielded qualitatively similar results.

Calculation of d' measure

We calculated a measure of discrimination (d') and bias (C) for participant scores on the word recognition and object recognition tasks (Snodgrass and Corwin, 1988). In each task, we calculated the fraction of correct responses to old objects out of all old object responses as hits (H), and the fraction

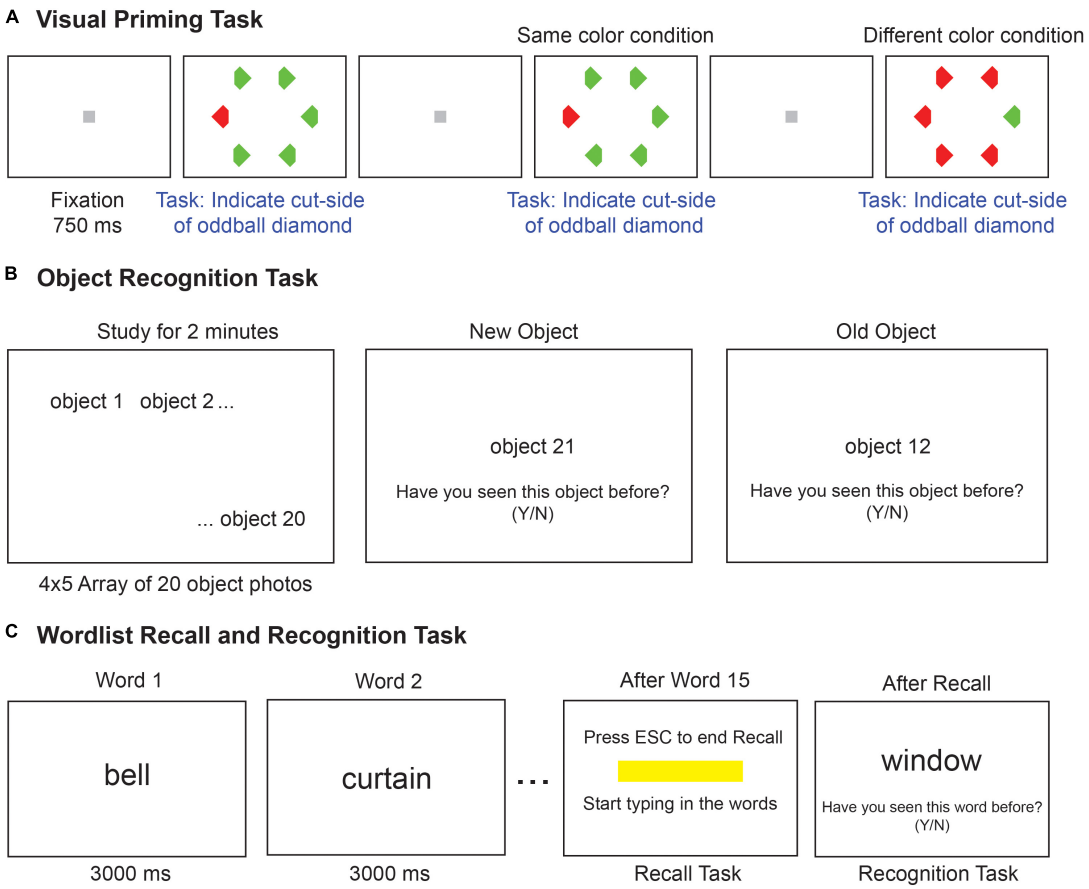

FIGURE 1 | Implicit and explicit memory tasks. **(A)** Schematic of the visual priming task with its two possible conditions. Each trial started with a gray fixation square followed by a search array. The second trial shows the odd–colored target with the same color (*red*) as the previous target, hence it is the same-color condition. The next trial shows the case where the target color (*green*) is different from the previous trial (*red*), hence it is the different-color condition. Participants make faster responses on same-color trials compared to different-color trials, indicative of an implicit memory. **(B)** Schematic of the object recognition task. Participants were asked to study a set of 20 common object photographs for 2 minutes. In the test phase (second and third panels), one picture was shown at a time and participants had to indicate whether the object was shown or not shown during the study phase. **(C)** Schematic of the word recall and recognition tasks. During the study phase (*left panel*), 15 words are presented for 3 s each in a sequence. In the recall phase (*middle panel*), a text box appeared on the screen, and the experimenter typed in the words recalled verbally by the participant. In the recognition task (*right panel*), 30 words were presented in sequence, and participants had to indicate whether the word was shown or not during the study phase.

of incorrect responses to new objects out of all new object responses as false alarms (FA). To avoid infinite values arising from H = 1 or FA = 0, we reduced these numbers by 1/N where N is the total number of trials (N = 40 for object recognition, N = 30 for word recognition tasks). The d' measure was then calculated as $d' = z(H) - z(FA)$, where the function $z(x)$ refers to the inverse cumulative distribution function of a standard normal distribution at the value x. The bias measure was calculated as $C = -(z(H) + z(FA))/2$. A positive bias implies that participants avoided false alarms at the expense of misses (i.e., avoided declaring new items as old).

RESULTS

Our goal was to characterize explicit and implicit memory across age and cognitive impairments. We compared these measures between young vs older volunteers and between MCI patients and age- and education-matched controls. The tasks performed by each participant are detailed in **Figure 1**.

In the implicit perceptual priming task (**Figure 1A**), participants had to report the shape of an oddball item in a hexagonal search array while its position or color was varied independently. As a result, any response speedup due to repeated color or position is independent of influences from making repeated motor responses (i.e., independent of motor priming).

We compared the performance on this implicit perceptual priming task with three explicit memory tasks. In the object recognition task (**Figure 1B**), participants were asked to study an array of objects and had to discriminate studied items from novel items. We selected this task because it is a measure of explicit visual object memory as opposed to verbal memory. In the word recall and recognition tasks (**Figure 1C**), participants were shown a series of words presented on a monitor for

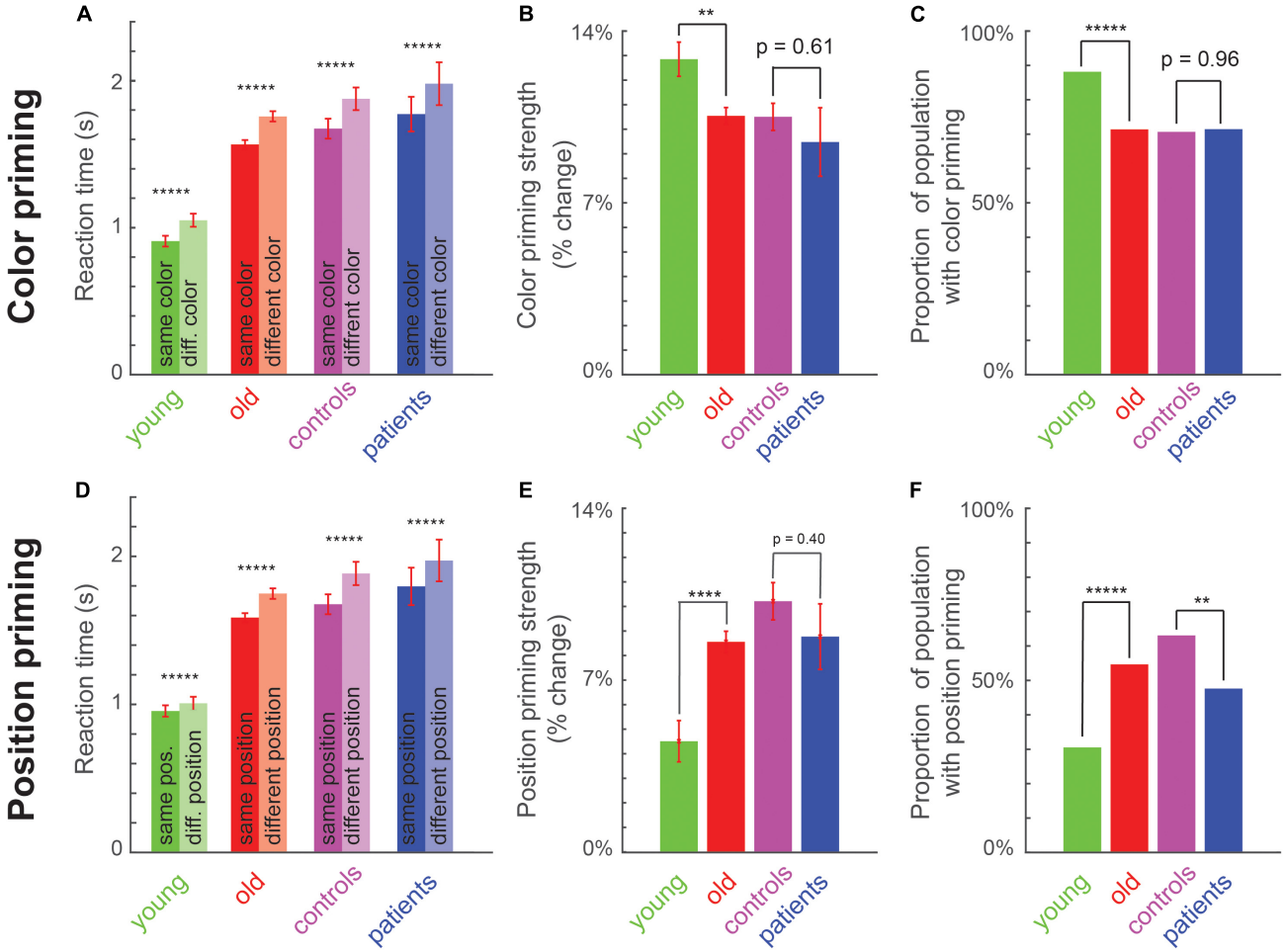

FIGURE 2 | Perceptual priming effects across age and cognitive impairments. **(A)** Average reaction time for same-color (dark) and different-color (light) trials across young, old, matched controls (am old) and patients. Asterisks indicate statistical significance of the main effect of color in an ANOVA (**Supplementary Section S2**): * is $p < 0.05$, ** is $p < 0.005$, etc., Error bars indicate SEM across participants. **(B)** Color priming strength across groups. Asterisks indicate statistical significance comparing priming strength across participants between groups, using a rank-sum test (*$p < 0.05$, **$p < 0.005$, etc.). Error bars indicate SEM across participants. **(C)** Proportion of population with significant color priming shown for each group of participants. Asterisks indicate statistical significance comparing the rate of incidence between each pair of groups, using a chi-squared test, with conventions as before. **(D–F)** Same as **(A–C)** but for position priming.

3 s each, and were subsequently asked to recall these words (word recall task) or discriminate them from novel words (word recognition task).

Perceptual Priming Task

All four groups of participants were highly accurate on this task (accuracy, mean ± sd: 96 ± 5% for young, 97 ± 4% for old; 97 ± 4% for matched controls, and 95 ± 7% for MCI patients). Compared to young participants, older participants were generally more accurate (cohen's d = 0.4, z = 3.85, $p < 0.0005$, rank-sum test on overall accuracy for young vs. old), but were considerably slower (average RT, mean ± sd: 1.02 ± 0.34 s for young; 1.75 ± 0.56 s for old; cohen's d = 1.2, z = 9.66, $p < 0.00005$, rank-sum test). By contrast, compared to matched controls, patients were less accurate (z = 2.38, $p = 0.02$, rank-sum test) but equally fast (mean ± sd of RT: 1.97 ± 0.62 s, 1.86 ± 0.71 s, cohen's d = 0.16, z = 1.17, $p = 0.24$, rank-sum test). Thus, MCI patients slightly less accurate but similar in speed compared to matched controls.

To measure implicit memory, we compared the reaction time on trials preceded by a target of the same versus different color or position. In all four groups (young, old, matched controls and patients), participants were faster for same-color trials compared to different-color trials in all groups (**Figure 2A**), and were faster for same-position trials compared to different-position trials (**Figure 2D**). A detailed statistical comparison is provided in **Supplementary Section S1**.

Participants were also more accurate on the same-color trials compared to different-color trials in all groups (accuracy, mean ± sem for same and different color trials: 97% ± 0.6% and 93% ± 1% for young participants; 97.8% ± 0.2% and 96.5% ± 0.3% for older participants; 94.9% ± 1.5% and 94.5% ± 1.6% for MCI patients; 97.6% ± 0.3% and 96.4% ± 0.5% for matched controls). Likewise, participants were more accurate

on same-position trials compared to different position trials for each group (accuracy, mean ± sem for same and different position trials: 97.3% ± 0.6% and 94.9% ± 0.7% for young; 98% ± 0.2% and 96.3% ± 0.3% for older participants; 95.4% ± 1.6% and 93.9% ± 1.6% for MCI patients; 97.8% ± 0.4% and 96.2% ± 0.5% for matched controls). Thus, the faster responses in the same-color and same-position trials cannot be explained as a speed accuracy tradeoff.

Thus, priming of pop-out is robustly present at the group level in young, old, matched controls and patients.

Do Color and Position Priming Vary With Age or With Cognitive Impairment?

Next we asked whether the strength of priming was different across age or with cognitive impairment. To this end, we calculated the priming strength for each participant as the percentage change in reaction time between primed (i.e., same color or position) and unprimed (different color or position) trials. Calculating the percentage change ensures that the measure is normalized to the speed of each participant and therefore comparable across participant groups.

To ascertain the reliability of variations in priming strength across participants, we calculated the priming strength using odd and even-numbered repetitions for each participant, and asked whether the two measures were correlated across participants. This split-half correlation, which is an index of reliability, was moderate in magnitude and statistically significant for both color and position priming strengths (r = 0.43 for color priming and 0.55 for position priming, $p < 0.00005$ in both cases; calculated across the older group). However, we note that these correlations do not reflect the correlation between participants that would be obtained on repeating the entire experiment (i.e., the test-retest reliability), since they are based on comparing two halves of the data. To estimate the expected test-retest correlation, we performed a Spearman-Brown correction given by $rc = 2r/(1 + r)$, where r is the split-half correlation. These estimated test-retest correlations were large in both cases, suggesting that the priming strength measures are robust across participants (r = 0.60 and 0.71 for color and position priming).

Next we examined differences in color and priming strengths across groups. Color priming strength was significantly weaker for older participants compared to young participants (**Figure 2B**; Priming strength: 13 ± 1% for young, 11 ± 0% for old, cohen's d = 0.41, z = 2.77, $p < 0.05$, rank-sum test). It was numerically weaker for patients compared to matched controls, but this effect was not statistically significant (**Figure 2B**; Priming strength: 9 ± 1% for patients, 11 ± 1% for matched controls, cohen's d = 0.19, z = 0.51, $p = 0.61$, rank-sum test). By contrast, position priming was stronger in older participants compared to young (**Figure 2E**; Priming strength: 5 ± 1% for young, 8 ± 0% for old, cohen's d = 0.56, z = 4.25, $p < 0.0005$, rank-sum test). As with color priming, position priming was numerically weaker in patients compared to matched controls but this effect was not statistically significant (Priming strength: 9.1 ± 0.7% for patients, 10 ± 1% for matched controls, cohen's d = 0.2, z = 0.89, $p = 0.37$, rank-sum test).

The variations in color and priming strength between young and old participants may indicate a general change in priming strength with age, or a selective change in a subset of participants. To explore these possibilities, we performed a participant-wise analysis to detect the presence of color or position priming. For each participant, we performed an ANOVA on the response times, with color, position and motor priming as factors. The fraction of participants with a significant color priming effect in each group is shown in **Figure 2C**. Color priming was less prevalent in old compared to young participants (**Figure 2C**; Percentage of participants with significant color priming: 88% for young, 71% for old, $\chi^2 = 70.67$, $p < 0.000005$, chi-squared test comparing young participants with and without color priming against the numbers predicted using the incidence in the smaller group, i.e., young participants; $\chi^2 = 7.31$, $p = 0.007$ for the same test with incidence predicted using the larger group). It was also less prevalent among patients compared to matched controls, but this difference was not statistically significant (**Figure 2C**; Percentage of participants with significant color priming: 71.4% for patients, 70.6% for matched controls, $\chi^2 = 0.03$, $p = 0.87$, chi-squared test using incidence predicted from smaller group; $\chi^2 = 0.002$, $p = 0.96$).

By contrast, position priming was more prevalent among old compared to young participants (**Figure 2F**; Percent of participants with significant position priming: 31% for young, 54% for old, $\chi^2 = 73$, $p < 0.000005$, chi-squared test using incidence predicted from the smaller group; $\chi^2 = 12.9$, $p < 0.0005$ using the larger group). It was also significantly less prevalent among MCI compared to matched controls (**Figure 2F**; Percentage of participants with significant position priming: 48% for patients, 62% for matched controls, $\chi^2 = 8.2$, $p = 0.0043$, chi-squared test using smaller group; $\chi^2 = 1.53$, $p = 0.22$ using the larger group). We obtained qualitatively similar results upon selecting participants with position priming strength above a threshold.

Finally, we asked whether participants exhibited differences in motor priming, i.e., whether they were faster when they had to make the same motor response (indicating the cut-side of the diamond) on consecutive trials, compared to when they had to make a different motor response. Motor priming did not differ in strength between young and old participants (priming strength: -1 ± 1% for young, 0 ± 0% for old, cohen's d = 0.24, z = 1.5, $p = 0.09$, rank-sum test) or between patients and matched controls (priming strength: 0 ± 1% for patients, 0 ± 0% for matched controls, cohen's d = 0.13, z = 0.20, $p = 0.84$, rank-sum test). But its incidence was relatively low across participants and decreased with age (percent of participants with significant motor priming: 14% in young, 7% in old, $\chi^2 = 9.1$, $p < 0.005$, chi-squared test using incidence from smaller group; $\chi^2 = 2.9$, $p = 0.09$ using larger group). Motor priming was numerically less frequent in patients compared to controls but this trend was not statistically significant (percent of participants with significant motor priming: 0% in patients, 8.7% in matched controls, $\chi^2 = 2.3$, $p = 0.13$, chi-squared test using smaller group; $\chi^2 = 0.06$, $p = 0.8$ using larger group; assuming non-zero prevalence in patients). Thus, motor priming is weakly present in general but declines across age.

In sum, we conclude that implicit perceptual priming for color and position show differential effects across age: Older participants show weaker color and motor priming but stronger position priming compared to young subjects. Position priming was less prevalent among MCI patients compared to matched controls.

Explicit Memory Differences

The word recall and recognition tasks used in our study involved making computer-based responses as well as viewing study words visually and recalling them verbally. To be sure that performance on these tasks is similar to the more standard verbally administered word recall and recognition tasks, we asked whether participants performance was correlated between our tasks and an independently administered word recall and recognition task from the Tata Longitudinal Study of Aging, in which an independent word list was used and words were presented purely verbally. This revealed a significant positive correlation (correlation coefficient across older volunteers: immediate word recall: r = 0.37, $p < 0.000005$ and immediate word recognition: r = 0.15, $p = 0.052$). The relatively low correlation may be due to the difference in presentation versus test modality in our task (Schacter, 1992; Schacter et al., 1993).

Next we investigated whether explicit memory differs across age and cognitive impairments. Object recognition memory was significantly weaker in old compared to young participants (**Figure 3A**; average accuracy: 92 ± 1% for old, 96 ± 1 for young, cohen's d = 0.49, z = 2.62, $p < 0.05$, rank-sum test) and in patients compared to matched controls (**Figure 3A**; average accuracy: 84 ± 1% for patients, 91 ± 1 for matched controls, cohen's d = 0.89, z = 3.31, $p < 0.005$, rank-sum test).

Likewise, word recall was significantly worse for old compared to young participants (**Figure 3B**; average percentage of words recalled: 45 ± 1% for old, 55 ± 2 for young, cohen's d = 0.6, z = 3.91, $p < 0.0005$, rank-sum test). Patients showed weaker word recall compared to matched controls, but this effect was not significant (average percentage of words recalled: 39 ± 4% for patients, 42 ± 2 for matched controls, cohen's d = 0.21, z = 0.95, $p = 0.34$, rank-sum test).

Finally, word recognition was weaker for old compared to young participants (**Figure 3C**; average accuracy on old/new word recognition: 87 ± 1% for old, 92 ± 2 for young, cohen's d = 0.63, z = 2.91, $p < 0.005$, rank-sum test). Patients showed significantly worse word recognition compared to controls (**Figure 3C**; average accuracy: 80 ± 2% for patients, 87 ± 1 for matched controls, cohen's d = 0.63, z = 2.45, $p < 0.05$, rank-sum test).

To confirm that the same trends are present using other performance measures, we calculated a d' measure of performance for the two recognition tasks, as well as their response bias (see Methods). In the object recognition task, older participants had a significantly smaller d' compared to younger participants (d', mean ± sem: 3.7 ± 0.2 for young, 3.28 ± 0.17 for old, cohen's d = 0.52, z = 2.43, $p < 0.05$, rank-sum test), and patients had smaller d' compared to matched controls (d', mean ± sem: 2.27 ± 0.08 for patients, 3.08 ± 0.14 for matched controls, cohen's d = 0.87, z = 3.6, $p < 0.0005$, rank-sum test). However, we observed no systematic differences in response bias (C, mean ± sem: −0.09 ± 0.04 for young, −0.03 ± 0.02 for old, cohen's d = 0.15, z = 0.92, $p = 0.36$, rank-sum test; −0.04 ± 0.08 for patients, −0.02 ± 0.04 for matched controls; cohen's d = 0.04, z = 0.4, $p = 0.69$).

In the word recognition task, older participants had significantly smaller d' compared to young participants (d', mean ± sem: 1.66 ± 0.1 for young, 1.39 ± 0.03 for old, cohen's d = 0.55, z = 2.6, $p = 0.009$) and patients had smaller d' than matched controls (d', mean ± sem: 1.15 ± 0.09 for patients, 1.39 ± 0.05 for controls, cohen's d = 0.5, z = 2, $p = 0.042$). However, we observed no systematic difference in response bias (C, mean ± sem: 0.89 ± 0.04 for young, 0.83 ± 0.01 for old, cohen's d = 0.24, z = 1.2, $p = 0.22$; 0.83 ± 0.07 for patients, 0.83 ± 0.03 for controls, cohen's d = 0.01, z = 0.2, $p = 0.84$).

We conclude that explicit memory measures are weaker in older participants compared to younger participants, and in patients compared to controls.

Relation Between Implicit and Explicit Memory

To investigate whether explicit and implicit memory covaried across participants, we calculated the pairwise correlation between implicit and explicit measures for each group. The resulting correlations are shown in **Figure 4**. We observed significant correlations only in older participants (**Figure 4B**), presumably because this was the group with the largest numbers. In this group, explicit memory measures were all highly correlated (**Figure 4B**). This is an interesting finding because the object recognition task involved simultaneously presented items with no control on item duration, whereas the word recall and recognition tasks involved items presented for fixed durations. The presence of a positive correlation implies that all three tasks are presumably governed by common explicit memory mechanisms.

By contrast, color and position priming strength were not significantly correlated (r = 0.08, $p = 0.22$; **Figure 4B**). Importantly, there was no significant correlation between explicit and implicit memory measures in both groups (**Figures 4A,B**).

We conclude that implicit and explicit memory show no covariation across participants.

Are Explicit and Implicit Memory Measures Consistent Across Follow-ups?

Since the older participants were participants in a longitudinal study, we were able to additionally assess whether participants showed reliable implicit and explicit memory scores across years. To do we simply asked whether the measures obtained in the first year (F0) were correlated with the same measures in the subsequent follow-up visit that occurred after about a year (F1). This revealed significant correlations for all measures (r = 0.44 and 0.50, $p < 0.005$ for color and position priming, respectively; r = 0.73 and 0.67, $p < 0.00005$ for word recall and recognition, r = 0.56, $p < 0.00005$ for object recognition; all correlations across 51 participants with F0 and F1 data). Thus, both explicit

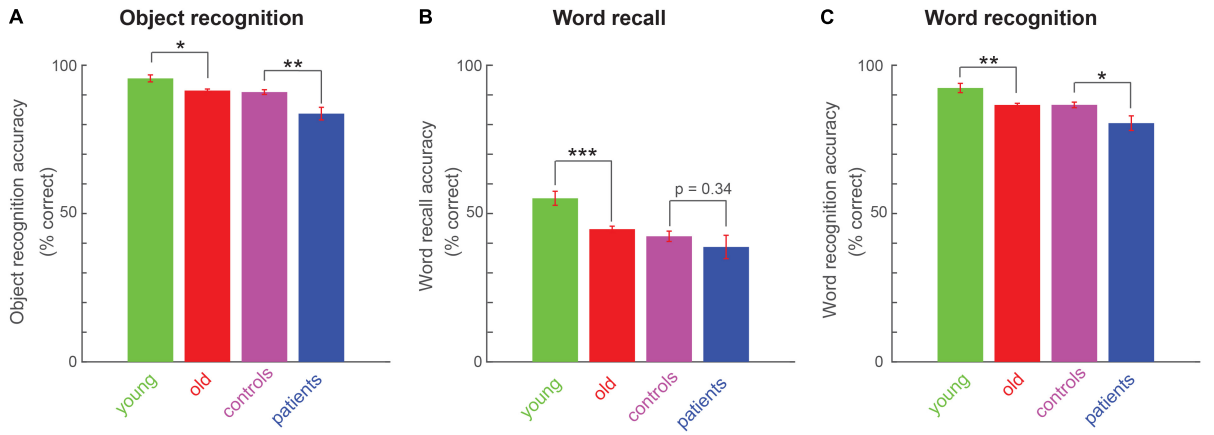

FIGURE 3 | Explicit memory variations across age and cognitive impairments. **(A)** Object recognition accuracy (percentage correct) across all participant groups. Asterisks indicate statistical significance comparing participant-wise accuracy using a rank-sum test. Conventions are as before. **(B)** Word recall performance (percentage correct) across all four participant groups, with conventions as in **(A)**. **(C)** Word recognition performance (percentage correct) across all four participant groups, with conventions as in **(A)**.

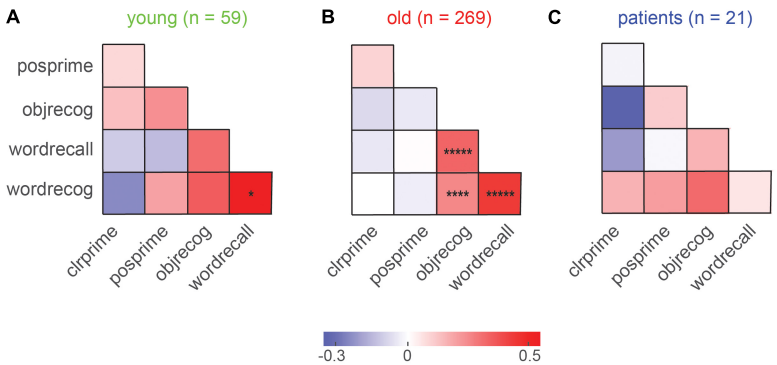

FIGURE 4 | Covariation in explicit and implicit memory across participants. **(A)** Pairwise correlation between explicit and implicit memory measures for young participants. Legends: *clrprime*: color priming strength, *posprime*: position priming strength, *objrecog*: object recognition accuracy, *wordrecall*: word recall accuracy, *wordrecog*: word recognition accuracy. Each entry indicates the Pearson's correlation coefficient between a given pair of memory measures across participants. Asterisks indicate statistical significance of these correlations (Conventions as before). **(B)** Same as **(A)**, but for older participants. **(C)** Same as **(A)**, but for MCI patients.

and implicit memory measures have a stable signature across longitudinal follow-ups.

DISCUSSION

Here we characterized implicit memory using perceptual priming and explicit memory across age and cognitive impairments. Our main findings are: (1) Explicit memory was weaker in old compared to young participants, and in MCI patients compared to age- and education-matched controls; (2) Surprisingly, perceptual priming did not always decline with age: color priming was smaller in older participants but position priming was larger; (3) Position priming was less frequent in the MCI group compared to matched controls; (4) Perceptual priming and explicit memory were uncorrelated across participants. These conclusions are based on cross-sectional comparisons, so they are consistent with (but do not directly prove) a longitudinal progression of priming with age or cognitive impairment. For instance, the differences could be due to cultural or generational factors. Below we discuss these findings in the context of the existing literature.

We have found that color priming is weaker in older participants. This is consistent with a decline in repetition priming observed previously in perceptual tasks (Soldan et al., 2009; Gordon et al., 2013; Caballero et al., 2018). Our findings are unlikely to be simply due to a decline in color discrimination with age (Paramei, 2012; Schneck et al., 2014), for several reasons. First, our task requires reporting the shape of an oddball target, although it does involve identifying the oddball by its color. Second, any participant impaired in color vision would show impaired performance on the task, but in fact both young and old groups were highly accurate on this task with very few exceptions (number of participants with below-80% accuracy:

1 of 59 for young; 2 of 269 for old; even these participants had accuracy > 65%). Third, even if such participants took longer to respond, their priming strength will remain unchanged since it is a normalized measure. Fourth, among the older participants (on whom a standard color blindness test was performed), only 2 were color blind. Even these two color blind participants performed our priming task at above 95% correct, presumably because they could use luminance information alone to find the oddball target. Finally, both color discrimination and color priming might be influenced by common cognitive factors such as attention that themselves decline with age or cognitive impairments (Fernandez-Duque and Black, 2006; McDonough et al., 2019). A deeper understanding of these issues will require comparing all these properties across age and cognitive impairments.

Our finding that position priming is stronger in older participants is novel and noteworthy since most cognitive measures decline with age. What could be the underlying mechanism? One possibility is that position priming is stronger in older participants because of their longer response times (Berry et al., 2017). However, our measure of priming is normalized to each participants baseline, so it is unlikely to be affected. Moreover the longer viewing times should have led to increased color priming, but we observed the opposite pattern. Alternatively, we propose that position priming, which is the facilitation of a previously viewed position, might require overcoming a competing mechanism, namely inhibition of return (IOR) by which recently viewed locations are suppressed (Posner, 2016). Whether this is really the case remains to be established since IOR is not known to operate across time intervals spanning ∼2 s across consecutive trials such as in our study. It is also well known that inhibitory control reduces with age (Lawrence et al., 2018). We therefore propose that the loss of inhibitory control leads to decreased inhibition of return, making it easier to orient to recently detected stimuli, in turn resulting in increased position priming with age. We have also found that position priming was less prevalent among MCI participants, suggesting that it can be a useful indicator of pathological aging. Thus, our findings can be explained if both the facilitation and inhibition of previously viewed locations are differentially affected by aging and cognitive impairments. These possibilities will require further study.

Here we tested participants from an urban, highly literate background, who were all familiar with the objects and words being used for the explicit memory tests. However, participants unfamiliar with the objects or words tested could easily show biased explicit memory measures due to novelty effects (Schacter et al., 1993). This is particularly important in the Indian context and perhaps even more so in clinical settings, where participants come from a wide variety of backgrounds (urban/rural and literate/illiterate), with heterogeneous levels of visual and verbal experience. These issues can be addressed by developing standardized measures for the Indian context (Iyer et al., 2020), and by tracking cognitive measures longitudinally.

Finally, we have found that perceptual priming is uncorrelated with explicit memory across individuals. This lack of correlation between implicit and explicit memory could be due to differences in stimulus type or modality (Schacter et al., 1993; Constantinidou and Baker, 2002), nature of the task or response (Fleischman and Gabrieli, 1998; Park et al., 1998; Mitchell and Bruss, 2003; Fleischman et al., 2005; Martins and Lloyd-Jones, 2006; Gong et al., 2010; Deason et al., 2015), or because of differently probing a common memory mechanism (Berry et al., 2008; Reber, 2013; Ward et al., 2013). Alternatively, explicit and implicit memory may be dissociable (Gazzaniga et al., 2014). We therefore propose that characterizing both explicit and implicit memory measures in studies of aging or cognitive impairment can yield a deeper understanding of memory systems than measuring either measure alone.

DATA AVAILABILITY STATEMENT

All data required to reproduce the results will be made available upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Human Ethics Committee, Indian Institute of Science, Bangalore. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KZ, SS, AM, and SPA designed experiments. KZ and SS collected data. RG, SP, and NR coordinated the cognitive testing of older participants. KZ and SPA wrote the manuscript with inputs from all other authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.576922/full#supplementary-material

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ORIGINAL RESEARCH
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Cognitive and Psychosocial Outcomes of Self-Guided Executive Function Training and Low-Intensity Aerobic Exercise in Healthy Older Adults

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Objectives: Prior work has demonstrated that executive function training or physical exercise can improve older adults' cognition. The current study takes an exploratory approach to compare the feasibility and efficacy of online executive function training and low-intensity aerobic exercise for improving cognitive and psychosocial functioning in healthy older adults.

Method: Following a standard pretest-training-posttest protocol, 40 older adults (aged 65 and above) were randomly assigned to an executive function or a physical training group. A battery of cognitive and psychosocial outcome measures were administered before and after training. During the 10 weeks of self-guided training at home (25–30 min/day, 4 days/week), the executive function training group practiced a set of adaptive online executive function tasks designed by Lumos Labs, whereas the physical training group completed an adaptive Digital Video Disc (DVD)-based low-intensity aerobic exercise program.

Results: Training transfer effects were limited. Relative to low-intensity aerobic exercise, executive function training yielded cognitive improvement on the 64-card Wisconsin Card Sorting Task (WCST-64), a general executive function measure. Depression and stress levels dropped following both training programs, but this could be driven by decreased stress or excitement in performing the tasks over time.

Discussion: The results revealed limited cognitive benefits of the online executive function training program, specifically to a near transfer test of general executive control. Importantly, the current study supports the feasibility of home-based self-guided executive function and low-intensity physical training with healthy older adults.

Keywords: executive function training, aerobic exercise, executive functions, psychosocial functions, Lumosity, aging

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INTRODUCTION

With a rapidly aging population, there is a growing need to identify methods to attenuate age-related cognitive decline or enhance cognition in later life (Green et al., 2019). Substantial age-related declines have been observed in executive functions (i.e., high-order attention regulation skills involved in planning, flexible thinking, and self-control; Grady, 2012), which may be associated with age-related cognitive declines in processes such as working memory (i.e., the ability to temporarily store and manipulate information; Lustig et al., 2007). The literature on cognitive training is vast but inconclusive with mixed or limited results on whether it can improve cognition during aging (Simons et al., 2016). Nevertheless, prior work has revealed that older adults' cognitive performance could be maintained or even improved by cognitive training (Kelly et al., 2014) or physical exercise (Hillman et al., 2008).

Cognitive Training

Prior research has shown that older adults' cognition is somewhat malleable and may benefit from either ability-specific cognitive training in fluid abilities (i.e., the ability to think and solve problems independent of learning and education) such as reasoning and processing speed (Ball et al., 2002; Yang, 2011), or by engaging in a cognitively stimulating activity/lifestyle (e.g., educational attainment, active learning of a new skill; Stine-Morrow et al., 2008; Park et al., 2014). Cognitive training has typically shown hierarchical transfer effects (Zelinski, 2009; Wilkinson and Yang, 2015; Simons et al., 2016), with greater *near* transfer to tasks that tap into the same abilities as the training tasks than *far* transfer to tasks that assess other cognitive abilities or functional domains. Although very limited, cognitive far transfer effects have been revealed in older adults from various forms of executive function training (Jaeggi et al., 2010; Webb et al., 2018). Similarly, other work has shown that cognitively stimulating/engaging lifestyles can benefit fluid abilities (Stine-Morrow et al., 2008; Park et al., 2014). A systematic review of 52 cognitive training studies has further revealed that computerized cognitive training (CCT) programs show a small but positive effect for certain cognitive domains in healthy older adults (Lampit et al., 2014). In contrast, some other studies have shown that certain forms of CCT produce little generalization to everyday cognitive skills (Melby-Lervåg et al., 2016). However, little research has examined the feasibility and efficacy of self-guided online cognitive training, a highly accessible contemporary way of learning, with advantages in progress tracking, temporal/geographical flexibility, and instant reinforcement. Although online programs such as Lumosity have been challenged for their alleged benefits to cognition (Kable et al., 2017), some past research has shown that these programs may have beneficial effects for attention and memory (Hardy et al., 2011; Ballesteros et al., 2015).

Physical Training

Along with an established positive relationship between physical activity and cognition in humans or animals (Swain et al., 2012), prior epidemiological and intervention studies have documented the cognitive benefits of physical exercise in older adults. Epidemiological studies suggest that older adults who remain physically active are at a decreased risk for developing cognitive impairments (Younan, 2018). Intervention studies suggest that physical exercise could yield a broad range of cognitive benefits, particularly in executive functioning (Hillman et al., 2008; Smith et al., 2010). Promisingly, a systematic review showed that even low-intensity physical exercise was effective at improving physical and cognitive health for older adults (Tse et al., 2015). Although one meta-analysis showed a potential advantage of cognitive over physical training for improving executive functions in older adults (Karr et al., 2014), another meta-analysis showed equivalent cognitive benefits between cognitive and physical exercise (Hindin and Zelinski, 2012). However, the efficacy of cognitive and physical exercise interventions have not been directly compared and thus it is unknown which type of intervention would be more likely to enhance cognition in healthy older adults. The current study thus took an explorative approach to compare the feasibility of online executive function training (i.e., *Lumosity*) with low-intensity aerobic exercise for eliciting near and far transfer effects on cognitive and psychosocial functions in older adults.

Psychosocial Benefits

Little attention has been paid to the benefits of cognitive or physical training for older adults' psychosocial functioning (e.g., the ability to perform the activities of daily living, regulate emotion, and engage in relationships; Kelly et al., 2014). Addressing this question allows us to identify accessible and effective ways to promote older adults' wellbeing. Results from the Advanced Cognitive Training for Independent and Vital Elderly (ACTIVE) study suggested that speed training, but not memory or reasoning training, improved self-rated health and reduced risks for depression or declining quality of life (Wolinsky et al., 2009). Physical training, including low-intensity exercise, has also been shown to alleviate depression in young-old adults (Bridle et al., 2012; Tse et al., 2015), but not in old-old adults aged 80 and above (Ansai and Rebelatto, 2015). Taken together, the psychosocial benefits of cognitive and physical training are largely understudied, and the available evidence is mixed.

The Current Study

We explored the efficacy of an online executive function training program against a low-intensity aerobic physical exercise regime for improving cognitive and psychosocial functioning in healthy older adults. Low-intensity aerobic exercise was used considering its effectiveness (Tse et al., 2015), safety and feasibility as a self-guided home-based exercise regime for older adults. Following the hierarchical transfer taxonomy (Zelinski, 2009; Wilkinson and Yang, 2015), we examined cognitive near transfer effects against outcome measures that overlapped with the training tasks in structure/ability (i.e., executive function or working memory), cognitive far transfer effects against tasks tapping untrained cognitive abilities (i.e., speed and episodic memory), and psychosocial far transfer effects against distant tasks assessing depression, stress, anxiety, and everyday activities. In light of the cognitive (Yang, 2011; Sprague et al., 2019) and physical training

literature (Hillman et al., 2008), we predicted that executive function training would lead to small but positive cognitive near transfer effects (Lampit et al., 2014) while physical training might otherwise show a broader cognitive benefit. Based on prior work (Wolinsky et al., 2009; Bridle et al., 2012), it is further predicted that both training protocols might show psychosocial benefits.

To address these goals, healthy older adults were enrolled in an executive function or a low-intensity physical exercise training program, which required the completion of self-guided activities at home for 25–30 min/day, 4 days/week for 10 weeks. Self-guided training/exercise has been shown to be effective in eliciting cognitive benefits without noted adherence problems in older adults (Yang et al., 2006; Yang, 2011; Hindin and Zelinski, 2012). The executive function training group practiced a set of online executive function or working memory tasks designed by Lumos Labs,[1] whereas the physical training group completed an aerobic exercise program following a series of DVDs.

MATERIALS AND METHODS

Participants

Based on *a priori* power analysis using G*Power 3.1.9.2 (Faul et al., 2007), a sample of 38 participants would provide 85% power to detect the critical Group × Session interaction (which signals the transfer effect) with a medium effect size of $f = 0.25$ (corresponds to $\eta^2 = 0.06$). The final sample included 40 healthy older adults (aged 65–87; $M = 70.83$, SD = 5.25, see **Table 1**) and informed consent was collected before their participation. Participants were recruited from the Ryerson Senior Participant Pool (RSPP), a university-organized database of approximately 700 older adults.

They were first screened for eligibility through their database information and via phone screening to include those without: (1) severe medical conditions (e.g., uncontrolled diabetes and/or cholesterol, cardiovascular diseases) that might endanger their participation in physical fitness training; (2) previous neurological disorders including stroke, prolonged periods of unconsciousness, and head injury; (3) uncorrected vision or hearing problems; and (4) previous participation in a cognitive or physical training intervention within the past five years. Participants were also required to have access to the internet or a DVD player as well as prior experience navigating the internet. Participants that met these criteria were invited to participate and randomly assigned to the executive function or the physical training group. At the pretest session, participants were further screened for potential dementia-related cognitive impairment using the Mini-Mental Status Exam (MMSE; Folstein et al., 1975), and all of them scored above the cut-off score of 23. Those assigned to the physical training group also completed the Physical Activity Readiness Questionnaire for Everyone (PAR-Q +; Warburton et al., 2011). Based on their responses, those with health and medical conditions ($n = 6$) were asked to consult with a qualified health care practitioner or exercise professional for potential health and safety issues related to their participation before they start.

At pretest, all participants completed (1) the Modifiable Activity Questionnaire (MAQ; Kriska et al., 1997) for self-reported time in 40 physical activities over one month; (2) a lab-made Cognitive Activity Questionnaire (CAQ) for self-rated frequency of engagement in 12 cognitively stimulating activities using a 5-point Likert Scale, with '1' indicating 'once a year or less' and '5' meaning 'every day or about every day'; and (3) the Home Step Test (CSTF, 2020) for physical fitness, which requires stepping up and down on an exercise step for 3 min while heart rate is being recorded at baseline and immediately after. The two groups were similar on these variables (see **Table 1**).

Design and Procedure

This study adopted a standard pretest-training-posttest protocol using a 2 (training: executive function vs. physical) × 2 (session: pretest vs. posttest) mixed-model design, with training group as a between-subjects variable and session as a within-subjects variable.

Pretest and Posttest Sessions

A battery of cognitive and psychosocial outcome measures (**Table 2**) were administered at both the pretest and posttest sessions (approximately 3.5–5 hours each), within a week of the start and the completion date of the 10-week training schedule, respectively.

Training Sessions

Similar to previous work (Berryman et al., 2014), we adopted a short-term training schedule (i.e., 25–30 min/day, 4 days/week for 10 weeks). At each training session, the executive function group completed an online cognitive training course consisting of 10 Lumosity executive function tasks, which included five for the first 10 sessions, and then added one new task every 5 sessions (see **Supplementary Appendix A** for task descriptions). All tasks were adaptive to participants' individual performance level. At each session, the physical group completed an indoor aerobic exercise workout following a video clip from one of three DVD workout programs, which featured low-intensity exercise appropriate for older adults to do on their own: *Jane Fonda's Prime Time*, *Winnipeg in Motion*, and *Jane Fonda's Firm and Burn* representing easy, medium, and difficult intensity levels, respectively. Participants were instructed to start with the easy program and gradually progress to higher intensity levels based on their own performance and fitness level, targeting a minimum of 50% heart rate increase at each session. These DVD programs were selected considering their popularity and focus on low-impact aerobic exercise (Krucoff, 1990). The clips were reviewed, piloted, and selected by the research team to ensure age-appropriateness, safety, length, and feasibility for home-based exercise. Each session started with a brief warm-up period, followed by 25–30 min of aerobic exercise, and then by a cool-down period. Participants were given a heart-rate monitor watch to record their heart rate right before the warm-up (baseline), and after the aerobic exercise section but prior to cool-down (post-exercise) at each session.

[1] http://www.lumosity.com

TABLE 1 | Sample characteristics.

Variables	Executive function training (n = 20)	Physical training (n = 20)	Group difference
			p
Age (years)	72.25 (5.98)	69.40 (4.07)	0.086
Gender (male:female)	5:15	5:15	1.000
Formal education (years)	18.32 (2.52)	17.00 (2.70)	0.124
Health rating	8.35 (0.93)	8.75 (1.16)	0.232
MAQ (minutes)	24,687.40 (19,561.08)	31,586.20 (22,360.19)	0.306
CAQ (sum score)	39.15 (7.23)	42.90 (5.21)	0.068
MMSE	28.40 (1.70)	28.45 (1.54)	0.923
Home step test (number of steps)	53.53 (9.25)	61.00 (13.68)	0.069
Home step test (heart rate increase)	47.33 (19.78)	50.32 (17.81)	0.632

Most cells present the mean (M), with standard deviation (SD) in parenthesis, except for gender cells which present a ratio score. Between-group comparisons were made using separate independent t-tests apart from gender ratio, which was examined using Pearson's chi square. MAQ, the Modifiable Activity Questionnaire; CAQ, the Cognitive Activity Questionnaire; MMSE, the Mini-Mental State Examination. Sample size = 40 for all the comparisons, except for education (n = 39), number of steps (n = 35), and heart rate increase (n = 37). Heart rate was assessed in beats per minute (BPM) and the heart rate increase was calculated by subtracting the baseline BPM from the BPM right after the Home Step Test.

A training log was also completed at each training session to record time, heart rate readings, and note any problems or general comments on the training tasks. The log also included weekly activity tracking in which participants recorded the time (in minutes) they spent in various cognitive (e.g., reading, writing, gaming) or physical (e.g., jogging, swimming, dancing) activities outside the training program. To check on progress and address questions, participants were called three times a week. Training completion was monitored through Lumos Labs' data, heart rate recordings, and training logs. Based on the daily training logs, the average adherence rate (i.e., percentage of sessions completed) was 93.88% (91.63% for the executive function group and 96.13% for the physical group). More than 80% of participants completed over 90% of the training sessions.

Outcome Measures

Cognitive Near Transfer Tasks

Cognitive near transfer tasks included those that were structurally similar to or taxed the same abilities (e.g., executive function or working memory) as the training tasks (**Supplementary Material: Appendix A**).

The Digit N-Back Task

The digit *N-Back task* (Wilkinson and Yang, 2016a) is an executive function task that taps into updating abilities. Participants viewed sequentially presented single digits (1–9) and indicated via key press whether each digit matched a pre-specified target (0-back), the digit presented immediately before (1-back), or two trials before (2-back). There were three blocks of trials, each including 10 practice trials followed by 45 test trials (including 9 target trials). Participants pressed the z key, labeled as "TARGET," and the / key, labeled as "NON-TARGET," counterbalanced across participants, as fast and accurately as possible. Two parallel versions of the task were counterbalanced across the pretest and the posttest sessions, with different digit sequences and target stimuli (i.e., 5 or 7 in the 0-back block). The dependent variables included hit rate (i.e., the proportion of targets correctly identified), false alarm (FA) rate (i.e., the proportion of non-targets misidentified as targets), and reaction time (RT).

The Stroop Task

The *Stroop task* (Stroop, 1935; Wilkinson and Yang, 2016b) is an executive function task that utilizes inhibition. Participants viewed single words and indicated the ink color of the word by pressing corresponding keys on the keyboard as fast and accurately as possible. They completed 280 trials (including 64 practice trials), which included an equal proportion of three trial types: congruent (e.g., the word "BLUE" printed in blue ink), incongruent (e.g., the word "BLUE" printed in green ink), and neutral (e.g., "XXXX" printed in blue ink). Two parallel versions of the task were counterbalanced across the pretest and the posttest sessions, using different sets of colors (green, purple, blue, and orange in Set 1 and orange, yellow, pink, and green in Set 2). Following previous practice (Wilkinson and Yang, 2016a), the dependent variable was the Stroop interference ratio score, calculated by dividing mean RTs or accuracy (i.e., hit rate) of incongruent trials by that of neutral trials (i.e., RT interference ratio score = $RT_{incongruent}/RT_{neutral}$; Accuracy interference ratio score = $Hit_{incongruent}/Hit_{neutral}$).

The Navon Task

The *Navon task* (Navon, 1977) is an executive function task that utilizes response switching and interference resolution. Participants responded to the global or the local features of a series of compound letter stimuli. Following 16 practice trials, there were two blocks of 144 test trials (72 local and 72 global), which included two trial types: congruent trials with the two local and global dimensions matched (e.g., a large letter H composed of small letter Hs) and incongruent trials with the two dimensions mismatched (e.g., a large letter S composed of small letter Hs). Global and local trial types were intermixed within blocks and thus participants needed to switch the response dimensions from trial to trial. At the start of each trial, a cue signaled which feature to respond to, with a large rectangle cueing a global response and a small rectangle cueing a local response trial. Two

TABLE 2 | Summary of pretest vs. posttest performance, transfer effects, and test-retest correlations on transfer tasks.

Measures	Executive function training		Physical training		Group × session interaction			*r*
	Pretest *M* (SD)	Posttest *M* (SD)	Pretest *M* (SD)	Posttest *M* (SD)	*P*	η_p^2	*BF*	
Cognitive near transfer: *N*-back								
0-back hit	0.98 (0.03)	0.97 (0.10)	0.94 (0.19)	1.00 (0.02)	0.140	0.06	0.75	−0.08
1-back hit[s]	0.83 (0.17)	0.93 (0.10)	0.86 (0.17)	0.92 (0.08)	0.547	0.01	0.36	0.41**
2-back hit	0.71 (0.18)	0.79 (0.12)	0.81 (0.17)	0.79 (0.15)	0.052	0.10	1.47	0.55**
0-back false alarm	0.01 (0.03)	0.02 (0.04)	0.03 (0.05)	0.01 (0.03)	0.123	0.06	0.82	0.34*
1-back false alarm[s]	0.05 (0.09)	0.04 (0.05)	0.06 (0.07)	0.03 (0.04)	0.402	0.01	0.36	0.70**
2-back false alarm	0.09 (0.05)	0.10 (0.05)	0.09 (0.07)	0.06 (0.04)	0.134	0.06	0.78	0.48**
Cognitive near transfer: Stroop								
Accuracy interference[g]	0.89 (0.23)	0.95 (0.11)	0.98 (0.03)	0.99 (0.03)	0.339	0.02	0.45	0.40*
RT interference (ms)	1.26 (0.12)	1.27 (0.18)	1.20 (0.10)	1.21 (0.12)	0.960	< 0.01	0.31	0.53**
Cognitive near transfer: Navon								
Local accuracy interference	0.16 (0.33)	0.03 (0.27)	0.04 (0.10)	0.06 (0.14)	0.199	0.04	0.61	−0.35*
Global accuracy interference[s]	0.25 (0.28)	0.11 (0.11)	0.15 (0.11)	0.14 (0.20)	0.058	0.09	1.36	0.29
Local RT interference (ms)	92.87 (164.53)	29.44 (387.36)	99.99 (104.97)	246.52 (863.53)	0.337	0.02	0.45	0.01
Global RT interference (ms)	247.82 (269.73)	220.67 (274.64)	219.63 (269.8)	574.74 (128.52)	0.452	0.02	0.39	0.39*
Cognitive near transfer: WCST-64								
Total correct[gs]	38.75 (10.98)	45.35 (10.17)	46.05 (9.29)	44.10 (11.59)	0.001**	0.25	32.47	0.68**
Perseverative responses	13.80 (8.36)	10.70 (8.05)	9.25 (7.48)	11.00 (6.76)	0.067	0.09	1.23	0.42**
Perseverative errors[gs]	12.40 (7.28)	9.50 (6.17)	8.40(5.99)	9.70 (5.65)	0.038*	0.11	1.82	0.49**
Non-perseverative errors[gs]	12.85 (5.94)	9.15 (4.77)	9.55 (4.89)	10.20 (7.12)	0.020*	0.13	2.94	0.47**
Conceptual level responses[gs]	31.25 (14.96)	39.60 (14.84)	41.20 (13.00)	38.80 (15.57)	0.016**	0.18	7.17	0.63**
Categories completed[gs]	2.05 (1.57)	2.65 (1.53)	2.85 (1.57)	2.65 (1.76)	0.044*	0.10	1.65	0.69**
Trials to complete first category[gs]	27.65 (20.61)	18.35 (15.89)	18.70 (12.51)	26.15 (21.58)	0.012*	0.16	4.32	0.31
Failure to maintain set	0.35 (0.67)	0.60 (1.10)	0.75 (0.97)	0.45 (0.60)	0.098	0.07	0.95	0.26
Learning to learn	1.68 (7.87)	−4.37 (6.43)	0.85 (7.30)	−1.51 (9.87)	0.963	< 0.01	0.42	0.09
Cognitive near transfer: change-detection								
1-target[s]	0.81 (0.11)	0.86 (0.12)	0.81 (0.11)	0.84 (0.10)	0.334	0.03	0.65	0.82**
1-target 2-distractor[s,gs]	0.80 (0.11)	0.86 (0.11)	0.81 (0.12)	0.83 (0.10)	0.042*	0.11	1.71	0.78**
3-target	0.66 (0.08)	0.68 (0.09)	0.66 (0.09)	0.67 (0.09)	0.469	0.01	0.57	0.76**
Cognitive far transfer: DSST								
# Correct solutions[g]	59.15 (13.74)	59.35 (14.34)	69.45 (17.86)	70.95 (16.70)	0.478	0.01	0.38	0.94**
Cognitive far transfer: HVLT-R								
Immediate recall	24.15 (5.04)	25.20 (6.65)	25.60 (4.74)	26.00 (5.70)	0.688	< 0.01	0.33	0.60**
Recall learning slope	1.55 (0.79)	1.48 (1.02)	1.75 (0.87)	1.55 (0.84)	0.702	< 0.01	0.33	0.33*
Delayed recall	8.05 (2.31)	8.85 (2.30)	9.15 (1.66)	9.20 (2.46)	0.313	0.01	0.47	0.46**
Retention	0.84 (0.18)	0.89 (0.13)	0.91 (0.13)	0.90 (0.15)	0.288	0.03	0.49	0.24
Recognition discrimination[g]	10.75 (1.41)	10.95 (1.50)	11.35 (0.81)	11.42 (0.69)	0.770	< 0.01	0.32	0.16
Psychosocial far transfer: DASS-21								
Depression[g,s]	6.70 (8.34)	4.10 (6.60)	2.30 (2.92)	1.60 (3.15)	0.181	0.05	0.64	0.74**
Anxiety	5.00 (6.10)	5.40 (6.68)	2.50 (5.10)	1.65 (4.02)	0.286	0.03	0.49	0.80**
Stress[g,s]	11.00 (7.09)	9.40 (6.90)	5.60 (5.37)	3.50 (4.44)	0.709	< 0.01	0.33	0.80**
Psychosocial far transfer: IADL								
Sum score	7.80 (0.52)	7.80 (0.92)	7.70 (0.73)	7.80 (0.41)	0.423	0.02	–	0.79**

*WCST-64, 64-card Wisconsin card sorting task; DSST, Digit Symbol Substitution Task; HVLT-R, Hopkins Verbal Learning Test-Revised; DASS-21, 21-item depression anxiety stress scales; IADL, instrumental activities of daily living scale. p and η_p^2 values are for the Group × Session interaction that signals the transfer effects. BF, Bayes factor (not calculated for IADL due to lack of variance at baseline). [g]Group effect was significant; [s]Session effect was significant; [gs]Group × Session interaction was significant; *p < 0.05, **p < 0.01.*

versions of the task, with different letter stimuli ("S" and "H" or "A" and "E"), were counterbalanced across the pretest and the posttest sessions. Participants indicated which letter (e.g., "S" or "H") was the target letter at a global or local dimension by pressing the corresponding keys (z or /), as fast and accurately as possible. The key assignment was counterbalanced across participants but kept consistent between pretest and posttest sessions. Following prior practice (Wilkinson and Yang, 2016a), interference scores were calculated as the difference between congruent and incongruent trials in both RT and errors for each

dimension (i.e., RT interference = $RT_{incongruent} - RT_{congruent}$; Accuracy interference = $Error_{incongruent} - Error_{congruent}$).

The Computerized 64-Card Wisconsin Card Sorting Task

The computerized *64-card Wisconsin Card Sorting Task* (WCST-64; Kongs et al., 2000) assesses general executive control (planning, reasoning, set switching, flexible thinking, and updating, etc.). Across 64 trials, participants matched a response card to one of four stimulus cards based on one of the three sorting rules (color, shape, or number). Responses were made by pressing the number keys (1–4), each corresponding to one of the stimulus cards. Participants were not informed of the correct sorting rule or when the rule shifted. The sorting rule was inferred via feedback ("Right" or "Wrong") following each response. Performance was indexed by nine variables: (1) *total correct* refers to the number of correct trials; (2) *perseverative responses* are cards continuously sorted, regardless of accuracy, according to a specific rule; (3) *perseverative errors* are cards continuously sorted according to a previous rule even after the rule has changed; (4) *non-perseverative errors* are other errors; (5) *conceptual level responses* are instances of three or more consecutive correct responses; (6) *categories completed* are instances of 10 consecutive correct responses; (7) *trials to complete the first category* are trials needed to successfully complete the first category; (8) *failure to maintain set* is the number of failures to continuously respond based on a correct sorting rule; and (9) *learning to learn* refers to the change in errors between successive categories.

The Change-Detection Task

The *Change-Detection task* (Jost et al., 2011) is a working memory task (i.e., capacity and distraction regulation specifically) in which participants were instructed to remember the orientation of target items (red rectangles) and ignore distracters (blue or green rectangles), that were presented as an array on either the left or right side of the screen. There were 120 trials equally split across three trials types, including "1-target," "3-target," and "1-target plus 2-distractors." Each trial began with an arrow cue directing participants to attend to the left or the right side of the screen. Following the testing stimulus array, a probe rectangle was presented and participants indicated whether the orientation of the probe was the same as the target item at the cued location by pressing the z or / keys, labeled as "yes" or "no," as fast and accurately as possible. The response key assignment was counterbalanced across participants. Performance was indexed by accuracy (i.e., percentage of correct responses) and RT.

Cognitive Far Transfer Tasks

Cognitive far transfer tasks assessed cognitive abilities that were not practiced during training. This included the *Digit Symbol Substitution Test* (*DSST*; Wechsler, 1981), a processing speed task in which participants substituted as many digits as possible with their corresponding symbols according to a provided digit-symbol mapping key. Participants first completed 7 practice trials followed by 133 trials in 2 min. The dependent variable was the number of correct completions. The *Hopkins Verbal Learning Test-Revised* (*HVLT-R*; Benedict and Hopkins, 2020) was used to assess verbal learning and memory. Participants learned 12 nouns from three semantic categories, followed by three trials of immediate recall (Trials 1–3). After 20 min, there was a delayed recall (Trial 4) and a yes/no recognition test including 12 lures (Trial 5). There were five dependent variables: (1) *total immediate recall* across Trials 1–3; (2) *immediate recall learning slope* (average gains per trial across Trials 1–3); (3) *delayed recall* (recall at Trial 4); (4) *retention* (Trial 4 divided by Trial 2 or 3, whichever was higher); and (5) *recognition discrimination* (hits minus FAs on Trial 5). No ceiling effects were noted (**Table 2**). Each of these tasks had two parallel versions, counterbalanced across the pretest and the posttest sessions.

Psychosocial Far Transfer Tasks

The *Depression Anxiety Stress Scale* (DASS-21; Lovibond and Lovibond, 1995) was used to assess depression, anxiety, and stress during the past week, which has a test-retest reliability ranging from 0.81–0.88 across the three subscales (Osman et al., 2012). Participants rated seven statements for each subscale using a Likert scale ranging from 0 ("did not apply to me at all") to 3 ("applied to me very much or most of the time"). Each of the depression, anxiety, and stress subscales were indexed by its own summed score, multiplied by 2. The *Instrumental Activities of Daily Living* (IADL) scale was used to assess functioning in eight daily living activities (i.e., ability to use telephone, shopping, food preparation, housekeeping, laundry, mode of transportation, responsibility for own medications, ability to handle finances), with a test-retest reliability of 0.80 (Lawton and Brody, 1969). For each activity, participants selected from a list of statements the one that most closely described their current level of functional ability (e.g., "Does personal laundry completely"). Each item was scored "1" if the ability could be performed at some minimal level of functioning or higher, otherwise, it was scored "0." The dependent variable was the total score, with a lower score indicating a higher dependence level.

Statistical Analysis

Data were analyzed in IBM SPSS 24. Significance level was defined at $a = 0.050$. Where necessary, Bonferroni corrections were modeled into the analyses to correct p-values for any exploratory multiple comparisons. Practice effects for each training task were assessed with a linear regression model for progressive changes in performance on executive function tasks or activity intensity of physical exercise across practice sessions. To test transfer effects, each dependent variable of the outcome measures was submitted to a 2 (Group: executive function vs. physical) × 2 (Session: pretest vs. posttest) mixed-model analysis of variance (ANOVA), with Group as a between-subjects variable and Session as a within-subjects variable. The transfer effect was indexed by the Group × Session interaction, with each group serving as a control for the other. To control for group baseline differences, variables/conditions that showed transfer effects were further analyzed on proportional training gain scores (gain from pretest to posttest divided by pretest). One-way ANOVAs were used to test group differences in training gain and one-sample *t*-tests followed to assess whether each group showed significant performance gains (i.e., above zero) at posttest compared to pretest baseline. The interaction

(i.e., transfer effect) for each variable was further tested for robustness with Bayesian hypothesis testing in Jeffreys's Amazing Statistics Program (JASP) (Van Doorn et al., 2019). Bayesian analysis confirms the likelihood of the presence or absence of an effect (alternative vs. null hypothesis) as indexed by a Bayesian factor (BF), with a BF = 3–10 meaning a moderate effect, and a BF over 10 suggesting a strong effect.

RESULTS

Eight participants (two in the executive function and six in the physical group) dropped out due to time restraints and were then replaced. Based on the independent-sample *t*-tests, no attrition effect was detected in most demographic (i.e., age, education, health rating, MAQ and CAQ scores) and psychosocial variables (i.e., DASS-21 and IADL scores), as well as on the DSST and MMSE, $ps \geq 0.173$. They also did not statistically differ from the final sample in the number of steps, immediate recall, recall slope, and retention ($ps \geq 0.058$). However, drop-out participants did show slightly different physical and memory profiles relative to the final sample as evidenced by their lower heart rate increase after the Home Step Test ($p = 0.011$) and lower recognition discrimination in HVLT ($p = 0.023$).

Practice Effects

Executive function training task performance was indexed by the Lumosity Performance Index (LPI), a standardized score generated and recorded by the Lumos Lab's Server after each training session. Exercise intensity at each session was indexed by the heart rate reserve (HRR), the proportional increase of peak (post-exercise) relative to resting (baseline) heart rate, as assessed in beats per minute (BPM; Smith et al., 2010). To assess practice effects, a linear regression model was conducted with session number as the predictor and LPI/HRR as the outcome variable for each training task (**Table 3**). All executive function training tasks showed significant practice effects, with no apparent ceiling effect (R^2s ≥ 0.73, βs ≥ 4.14, $ps \leq 0.01$), but HRR did not show significant practice effect ($p = 0.65$). The average HRR was 40.21%, suggesting a light exercise intensity (Kramer et al., 2002).

Transfer Effects

Prior to analyzing the RT data, outliers were trimmed by removing trials that were ± 2.5 SDs from the mean within each condition. Initial analyses on RTs in the *N-Back* and the *Change-Detection* tasks did not reveal a significant interaction ($Fs \leq 2.46$, $ps \geq 0.126$), thus they were omitted for brevity. All transfer effects (i.e., the Group × Session interaction and its BF) and reliability (pretest-posttest correlations) for each dependent variable are reported in **Table 2**.

Cognitive Near Transfer

For the N-Back task, the ANOVAs on hit and FA rates for each condition revealed significant Session effects for the 1-back condition in both hit, $F(1,38) = 10.93$, $p = 0.002$, $\eta_p^2 = 0.22$, and FA rates, $F(1,38) = 5.85$, $p = 0.020$, $\eta_p^2 = 0.13$, with a higher hit and lower FA rate at posttest vs. pretest, indicating improvement in both indices. The critical Group × Session interactions were not significant ($ps \geq 0.052$).

The ANOVAs on the Stroop RT and accuracy interference scores revealed larger interference in accuracy for the physical ($M = 0.99$, SD = 0.03) than the executive function group ($M = 0.92$, SD = 0.14), $F(1,38) = 4.24$, $p = 0.046$, $\eta_p^2 = 0.10$; all other effects were non-significant ($ps \geq 0.111$). For the Navon task, the ANOVAs on RT and accuracy interference scores for each condition revealed a significant Session effect in the accuracy analysis for the global condition, $F(1,38) = 4.49$, $p = 0.041$, $\eta_p^2 = 0.11$, with reduced interference scores at posttest relative to pretest (i.e., improvement), but the critical Group × Session interaction did not reach significance ($p = 0.058$). The local condition analyses did not reveal any significant effects ($ps \geq 0.199$).

For the WCST-64 task, the ANOVAs revealed a significant Group × Session interaction for all dependent variables, $Fs \geq 4.34$, $ps \leq 0.044$, η_p^2s ≥ 0.10, except for perseverative responses ($p = 0.067$), failure to maintain set ($p = 0.098$), and learning to learn ($p = 0.963$). One-way ANOVAs on the proportional gain scores (**Figure 1**) confirmed the group differences in total correct, perseverative errors, conceptual level responses, and trials to complete the first category ($Fs \geq 5.75$, $ps \leq 0.021$, $ds \geq 0.76$). The follow-up one-sample *t*-tests showed significant gains in total correct ($t = 3.90$, $p = 0.001$, $d = 0.87$) and conceptual level responses ($t = 2.52$, $p = 0.021$, $d = 0.57$) but not in other variables ($ps \geq 0.078$) following the executive function training. In contrast, physical training did not produce any significant gains ($ps \geq 0.064$). As a further validation, these variables were also analyzed by artificially matching the samples based on the baseline "total correct" range (i.e., 36–55), resulting in 12 participants in the executive function training and 17 in the physical training group, without group differences in all dependent variables ($ps \geq 0.46$). The one-way ANOVAs on the proportional gain scores of these baseline-matched samples revealed significant group differences in the following variables: total correct, conceptual level responses, and perseverative errors ($ps \leq 0.05$). The one-sample *t*-tests showed significant training gains in both total correct and conceptual level responses ($ps = 0.02$) for the executive function training group, whereas the physical group did not show significant gains in any variables ($ps \geq 0.12$).

For the Change-Detection task, we excluded one executive function training participant due to low accuracy at pretest (0.07–0.23 correct across conditions). The ANOVAs on accuracy revealed significant pretest to posttest improvement for the "1-target" and the "1-target plus 2-distractors" conditions, $Fs \geq 12.26$; $ps \leq 0.001$, η_p^2s ≥ 0.24, but not for the "3-target" condition ($p = 0.191$). The Group × Session interaction was significant only for the "1-target plus 2-distractors" condition, $F(1,37) = 4.44$, $p = 0.024$, $\eta_p^2 = 0.11$, with a significant benefit from the executive function training, $t(18) = -3.56$, $p = 0.002$, $d = 0.77$, but not the physical training, $t(19) = -1.12$, $p = 0.278$, $d = 0.33$. The one-way ANOVAs on the proportional gain score failed to reveal a significant group difference ($p = 0.083$). The one-sample *t*-tests showed significant gains in the "1-target" condition for both groups ($ts \geq 2.74$, $ps \leq 0.013$, $ds \geq 0.62$), in the

TABLE 3 | Linear regressions on the prediction of session number to training performance.

	# of Sessions	Initial performance	Final performance	R^2	*F*	B	*p*
Executive function training							
Brain shift	40	310.60	863.8	0.97	1,055.39	13.27	< 0.001
Color match	40	300.00	729.38	0.96	903.07	10.86	< 0.001
Face memory workout	40	216.20	456.87	0.98	1,627.60	5.88	< 0.001
Lost in migration	40	311.75	630.00	0.96	965.27	7.38	< 0.001
Memory matrix	40	425.45	638.15	0.93	499.60	4.15	< 0.001
Disillusion	30	291.05	784.87	0.98	1,478.31	17.64	< 0.001
Follow the frog	25	264.56	525.71	0.96	540.31	10.07	< 0.001
Route to sprout	20	242.06	702.79	0.80	73.06	17.82	< 0.001
Observation tower	15	412.10	603.47	0.68	27.51	8.86	< 0.001
Pinball recall	10	301.06	485.86	0.73	21.69	15.94	= 0.002
Physical training	40	30.51	40.57	0.01	0.20	0.02	0.65

Executive function training task performance was indexed by Lumosity Performance Index (LPI). Physical exercise performance was indexed by heart rate reserve (HRR). Please see ***Supplementary Appendix A*** *for the detailed description of each executive function training task.*

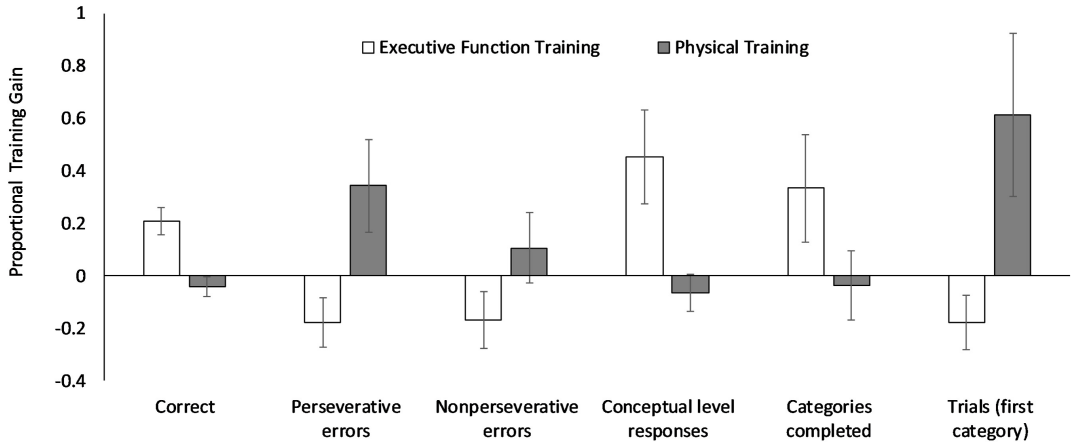

FIGURE 1 | The proportional training gain scores in the 64-card Wisconsin Card Sorting Task (WCST-64). Positive values mean the proportional score increases at posttest relative to pretest, with higher values meaning larger gains. Error bars refer to the standard errors of the means. Trials (first category) = trials to complete the first category.

"1-target plus 2-distractors" condition for the executive function group, $t(18) = 3.49$, $p = 0.003$, $d = 0.76$, but not the physical group ($p = 0.182$). Neither groups benefited in the "3-target" condition ($ps \geq 0.165$).

Cognitive Far Transfer

The ANOVA on the total number of correct responses on the DSST revealed a significant effect of Group, $F(1,38) = 5.00$, $p = 0.031$, $\eta_p^2 = 0.12$, with a higher score in the physical ($M = 70.20$, $SD = 17.10$) than the executive function group ($M = 59.25$, SD = 13.69), $d = 0.71$. All other effects were not significant ($ps \geq 0.354$).

For the HVLT-R, the ANOVAs on the five dependent variables outlined in the Methods section did not reveal any significant effects ($ps \geq 0.060$).

Psychosocial Far Transfer

For the DASS-21, the ANOVAs on the depression, anxiety, and stress scores revealed a significant main effect of Group ($Fs \geq 4.24$, $ps \leq 0.046$, $\eta_p^2 s \geq 0.10$) in depression and stress, with lower scores in the physical than the executive function training group (**Table 2**). The Session effect suggested decreased depression and stress after training, $Fs \geq 5.60$, $ps \leq 0.023$, $\eta_p^2 s \geq 0.12$. The interactions were not significant ($ps \geq 0.181$).

The ANOVA on the IADL score did not reveal any significant effects ($ps \geq 0.423$).

Summary

Taken together, results suggest that executive function training yielded positive cognitive near transfer effects to the WCST-64 relative to the physical training. Bayesian analysis also confirmed these transfer effects by showing a moderate to strong effect in three important variables of the WCST-64 (BF = 4.32–32.47). Additionally, depression and stress levels dropped following both training programs. Limited or no transfer effects were observed for any other cognitive or psychosocial outcome measures.

DISCUSSION

This study compared the cognitive and psychosocial benefits of executive function and low-intensity physical training programs in healthy older adults. Replicating previous findings (Wilkinson and Yang, 2012, 2016a; Yang et al., 2006), there was a significant practice effect on all executive function training tasks, validating the feasibility and efficacy of this program for eliciting practice effects. Compared to the low-intensity physical training program, executive function training also yielded positive, though limited, cognitive near transfer effects to the WCST-64, a measure of general executive control. No transfer effects were found to untrained cognitive abilities or daily living functions. Interestingly, depression and stress levels dropped following both training programs.

Cognitive Transfer Effects

The current findings provide evidence that self-guided online executive function training can produce near transfer effects to a general executive control task (i.e., WCST-64) but little far transfer to untrained cognitive abilities. This is consistent with a recent meta-analysis showing that the effects of cognitive training tend to be specific and do not generalize to other real-world cognitive skills (Melby-Lervåg et al., 2016). In line with the literature (Karr et al., 2014; Sprague et al., 2019), the current study revealed cognitive near transfer effects in the executive function training group as compared to the physical training group. The benefits are unlikely accounted for by training time or overall engagement level. The analysis on the logged training time at each session ($n = 13$ in the executive function and $n = 15$ in the physical group without missing data) showed that the physical group spent more time in training than the executive function group, particularly in the first 5 weeks of training ($ps \leq 0.001$). Furthermore, the self-reported weekly log of general cognitive and physical engagement outside of training, did not differ between the two groups or across weeks ($ps \geq 0.531$). Thus, training time and general engagement likely did not contribute to the near transfer effect in the executive function group.

One recent meta-analysis found no transfer of CCT to executive functions (Lampit et al., 2014), whereas others showed selective transfer of CCT to shifting and inhibition but not updating abilities (Webb et al., 2018) in healthy older adults. Unlike these findings, the current study showed limited transfer to ability-specific pure executive function tasks (i.e., N-Back, Stroop, Navon, and Change-Detection). One possibility is that the ability-specific nature of CCT in previous work may only elicit ability-specific benefits. The online executive function training used in the current study differs from CCT used in previous work in its accessibility, adaptability, and complexity. Most selected training tasks (e.g., *Route to Sprout* or *Pinball Recall*) engaged multiple executive functions or working memory skills. These features of the executive function training program in the current study may explain its limited or lack of transfer to ability-specific executive function tasks. It should be noted that both training programs improved performance on those less-challenging conditions (the 1-back block in the *N*-back task and the 1-target condition of the Change Detection Task), which may simply reflect a retest practice effect. Further research with a waitlist no-training control group is needed to rule out the retest practice effect.

Additionally, the transfer effects to the WCST-64 somewhat support transfers based on training abilities rather than task structure. The WCST-64 requires inferring and updating the sorting criteria (i.e., color, shape, and number of symbols) based on response feedback. The "Brain Shift" and "Disillusion" training tasks also require monitoring and shifting between response rules, but the rules were pre-set and provided to participants. Thus, the demonstrated near transfer to the WCST-64 likely suggests a general near transfer effect to the trained ability beyond the specific task structure, extending the results of non-item-specific retest learning (Yang et al., 2009). Additionally, the WCST-64 requires participants to continuously plan, reason, and update task rules and to map the rules to four motor key-pressing response options. Thus, responses to the WCST-64 task may be more dependent on manual dexterity (i.e., coordinated fine motor skills), which is strongly related to executive function in older adults (Kobayashi-Cuya et al., 2018). This may also account for the near transfer to the WCST-64.

No cognitive far transfer was detected to speed (DSST) or memory (HVLT-R). This finding is consistent with literature showing minimal or no cognitive far transfer effects following CCT (Yang et al., 2006; Owen et al., 2010; Wilkinson and Yang, 2016a), presumably because the training involves repeatedly practicing the same set of cognitive skills without learning any other new skills. Consistent with this idea, it has been found that age-related decline in complex cognition, such as reasoning and episodic memory, cannot be explained by executive function differences (Verhaeghen, 2011). However, the lack of the cognitive far transfer effects is inconsistent with some other studies (Zelinski, 2009; Jaeggi et al., 2010; Hindin and Zelinski, 2012). Further research is thus needed to identify factors underlying the previously reported cognitive far transfer effects.

Psychosocial Transfer Effects

Depression and stress levels dropped following both cognitive and physical training in the current study. These findings are consistent with earlier work showing reduced depression risk following cognitive training (Wolinsky et al., 2009) and alleviated depression following physical exercise (Bridle et al., 2012) and may suggest that exercise, whether cognitive or physical, has the potential to enhance certain psychosocial functions. We should note, however, that this benefit could be merely driven by a decrease in stress or excitement related to performing the training or transfer tasks over time. Again, given the exploratory nature of our study, further research with a no-training control group would help to rule out accounts related to reduced stress or excitement over time.

The lack of benefits on the self-reported IADL may be due to low variability and a ceiling effect, with an average score of 7.70–7.8 out of 8 across sessions and groups (**Table 2**). Nevertheless, the results are consistent with the ACTIVE study which found no immediate benefit on self-reported IADLs within the first 3 years of cognitive training, despite a long-term benefit after a 5–10-year delay (Rebok et al., 2014). However, we should note that

far transfer to everyday functions (including a timed IADL task) has been previously found with community-dwelling older adults following practice of a useful field of view task in a meta-analysis (Edwards et al., 2018).

Inconsistent with the literature (Hillman et al., 2008; Smith et al., 2010) and our hypothesis, physical training in this study produced little benefit. The improvement was expressed only on stress, depression, and two easy cognitive task conditions (the 1-back block in the *N*-back task and the 1-target condition of the Change Detection Task). This might be due to the low-intensity nature of the training (HRR = 40.21%; Kramer et al., 2002), though training was well maintained across sessions. Given the self-guided and home-based nature of training, we prioritized participants' safety and feasibility by choosing age-appropriate workouts (Nielsen et al., 2019), but they may not have been intensive enough to achieve the target HRR (e.g., 50–75%). Additionally, while executive function training was adapted to individual performance levels, the physical training group only had three options and thus it was not individually tailored. This may have restricted the potential benefits of the physical training program.

Limitations and Outlook

A number of limitations should be noted. First, the small sample size might have limited our ability to detect subtle effects. Although the power analysis indicated that our sample size had 85% power to detect a moderate Group × Session interaction ($f = 0.25$), caution should be taken in interpreting the transfer results (specifically those non-significant effects) based on merely hypothesis testing results. Second, despite random assignment, the physical group showed an overall better cognitive/psychosocial profile than the executive function group at baseline (on DSST, depression, stress, WCST-64 total correct and conceptual level responses, $ps \leq 0.048$). The smaller benefit of the physical group might be due to their higher baseline functioning, leaving little room for further improvement relative to the executive function group. However, it is important to note that the reported transfer effects (i.e., to the WCST-64) remained significant after the baseline group differences were controlled for in the analyses based on the proportional training gains and artificially baseline-matched samples. Furthermore, Bayesian analyses also confirmed the robustness of the transfer effects to the WCST-64. Third, the low-intensity nature of the physical exercise program might have restricted its potential beneficial effects, considering that some prior work has suggested that the cognitive benefits of physical exercise are intensity-dependent (Angevaren et al., 2007; Northey et al., 2018). Fourth, it is a challenge to make a fair comparison between the two programs as it is difficult to match the programs based on critical variables such as intensity level and quantitative outcome variables (e.g., behavioral index vs. heart rate change). To circumvent these challenges, we controlled for other important variables including the frequency and engagement time of the training sessions. As such, the results should be interpreted with caution in light of these challenges. Lastly, like some previous large-scale training studies (ACTIVE for example), the current study did not include an active or passive no-training control group to rule out retest practice effects. Nevertheless, the current study took an exploratory first step to evaluating and comparing the feasibility of using self-guided executive function and physical training programs with healthy older adults. Our results suggest a differential benefit of executive function training on a near transfer test of cognition, relative to the physical training group. The current study therefore adds value to the literature on behavioral interventions for improving older adults' cognition by highlighting potential differences in the effects of cognitive and physical training.

CONCLUSION

The results of the current study expand previous evidence for the efficacy of CCT in healthy older adults (Lampit et al., 2014). Specifically, the findings provide evidence, though limited, for the feasibility and efficacy of online executive function training for improving general executive function performance over and above a low-intensity physical exercise program in healthy older adults. These results add to the self-guided practice/training literature (Yang, 2011; Hindin and Zelinski, 2012) by validating that older adults can engage and adhere to a self-guided training program at home. Future research is required to identify the mechanisms underlying these transfer effects and to determine what factors may enhance the motivation and commitment of older adults to self-guided online cognitive training programs and thus maximize training benefits. For example, using multi-domain or combined training programs may be promising for far transfer effects to everyday functions.

DISCLOSURE STATEMENT

We confirm that all the behavior measures, manipulation conditions, data inclusion and exclusion procedures, and sample size determination approach involved in this study were reported.

DATA AVAILABILITY STATEMENT

The datasets generated in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: the data files and the related data file catalog file were deposited for open access to the Open Science Framework (https://osf.io/d7qrj/).

ETHICS STATEMENT

The project received ethics approval from the Ryerson Ethics Board (REB2013-061). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LY directed the design and operation of the project, conducted data analyses, and led the manuscript preparation. SG and LW

were involved in the actual operation of the project, including selecting and validating the physical training programs, selecting games for the executive function training program, training the research assistants, preparing testing packages, etc. BD was involved in programming and developing some cognitive outcome measures. All the authors contributed to the reviewing and editing of the earlier versions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.576744/full#supplementary-material

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Propofol Requirement and EEG Alpha Band Power During General Anesthesia Provide Complementary Views on Preoperative Cognitive Decline

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Background: Although cognitive decline (CD) is associated with increased post-operative morbidity and mortality, routinely screening patients remains difficult. The main objective of this prospective study is to use the EEG response to a Propofol-based general anesthesia (GA) to reveal CD.

Methods: 42 patients with collected EEG and Propofol target concentration infusion (TCI) during GA had a preoperative cognitive assessment using MoCA. We evaluated the performance of three variables to detect CD (MoCA < 25 points): age, Propofol requirement to induce unconsciousness (TCI at SEF_{95}: 8–13 Hz) and the frontal alpha band power (AP at SEF_{95}: 8–13 Hz).

Results: The 17 patients (40%) with CD were significantly older ($p < 0.001$), had lower TCI ($p < 0.001$), and AP ($p < 0.001$). We found using logistic models that TCI and AP were the best set of variables associated with CD (AUC: 0.89) and performed better than age ($p < 0.05$). Propofol TCI had a greater impact on CD probability compared to AP, although both were complementary in detecting CD.

Conclusion: TCI and AP contribute additively to reveal patient with preoperative cognitive decline. Further research on post-operative cognitive trajectory are necessary to confirm the interest of intra operative variables in addition or as a substitute to cognitive evaluation.

Keywords: EEG signal, general anesthesia (GA), brain age, alpha band power, cognitive decline and dementia

INTRODUCTION

Cognitive Decline (CD) is characterized by an impairment or a gradual weakening in cognitive functions such as memory, language, or judgment (American Psychiatric Association, 2013; Purdon, P. L., et al., 2015). Besides being common among elderly people, it is also associated with an increased risk to develop postoperative neurocognitive disorders (Greene et al., 2009; Lee et al., 2011; Silbert et al., 2015). These complications are linked to poor long-term outcomes including increased mortality, loss of autonomy and dementia (Inouye et al., 1998; Sprung et al., 2017). The estimated incidence-rates of preoperative CD might affect at least 24% of people over an age of 65 old (Culley et al., 2017). The increase in surgical procedures among the elderly, as a results of ageing population, has recently motivated the medical community to administer rapid neurocognitive tests prior to surgical intervention (Berger et al., 2018). In practice, such assessments are time-consuming and come with several pitfalls related to test administration. Moreover, stress and pain related to surgical procedure may systematically distort the measurements. Can the perioperative cognitive risk be objectively assessed without recourse to a patient or operator dependent scale?

Aging is a heterogeneous process leading to strongly individualized trajectories in cognitive functioning and health. Retrieving the date of birth from the patient's passport to determine cognitive risk is therefore not sufficient. This longstanding problem has early-on stressed the difference between chronological age and biological age (Chown, Dirken and Siegler in the '60s to '80s) and therefore the importance of research on specific measures of precocious and accelerated aging. In the past decade, the advent of machine learning in neuroimaging and biomedical research has led to burgeoning interest in proxy measures for individual aging. One such measure is the brain age, which is defined as the difference between the passport age and the age algorithmically predicted from population-level brain images (Dosenbach et al., 2010; Cole et al., 2019). Elevated brain age has been linked to various facets of cognitive dysfunction, morbidity and mortality (Liem et al., 2017; Cole et al., 2019). Recent studies suggest that brain age prediction can be enhanced by combining several neuroimaging modalities and found that electrophysiology adds unique information on aging (Liem et al., 2017; Engemann et al., 2020). First studies have demonstrated robust estimation of brain age from high-density EEG data (Sabbagh et al., 2020) and sleep EEG (Sun et al., 2019). However, this framework has been rarely applied outside of the laboratory. The simplification of the collection, the non-invasive character, the acceptance by the patient and the least cost are all criteria that could allow the optimization and generalization of the concept.

The EEG recorded during general anesthesia might provide a unique window on individual aging. During the intraoperative period, a systematic monitoring setup provides critical information on vital signals in real time. Among the per-operative arsenal used for patient monitoring, the EEG signal is often the method of choice to evaluate the depth of the sedation and subsequently adjust drug-administration. Some authors proposed a new complementary use of peroperative EEG aimed at predicting CD, or even more generally peri operative neurocognitive disorder, using patterns such as the intra operative Burst Suppression (BS) (Wildes et al., 2019). Recently, the decrease of power spectral density in the alpha band (8–13Hz), collected on the frontal EEG under general anesthesia has been associated not only to chronological age (Purdon et al., 2015) but also to preoperative CD (Koch et al., 2019). On the other hand, it is also recognized that the effective dosage of hypnotics under general anesthesia decreases with age (Schnider model and MAC) but also with preoperative cognitive status (Laalou et al., 2010). We previously presented evidence for an important association of sedation levels and EEG derived variables (Propofol requirement and Alpha band power) with chronological age and BS (Touchard et al., 2019). Yet, the precise relationship between these two variables and CD is still unclear. As relevant large-scale data necessary for machine learning is not available at this point, here we developed a classical statistical model to investigate potential biomarkers of cognitive decline based on standard monitoring data from general anesthesia.

The objective of this present study is to evaluate the relationship between intraoperative EEG, especially the alpha band power, effective Propofol dosage and the pre-operative CD evaluated using the Montreal Cognitive Assessment (MoCA). We propose an intraoperative model assessing indirectly cognitive decline under general anesthesia that we termed HELP: **H**ypnotic requirement and **E**EG signa**L** **P**ower.

METHODS

Patient Selection and Intra Operative Data Collection

Between November 2018 and May 2019, patients eligible for interventional neuroradiology or orthopedic surgery performed under general anesthesia were selected to participate in this prospective, observational, mono-centric study. Inclusion criteria were an elective surgery, a Propofol-based Total IntraVenous Anesthesia (TIVA), a French-speaking and adult (>18yo.) patient. Pregnant women, patients sedated under mechanical ventilation at the time of their management or with a BMI > 35 kg/m2 were excluded (TCI pharmacological model not applicable).

Frontal EEG data (Fp1, Fp2, F7, F8, a ground electrode at Fpz and a reference electrode 1 cm above Fpz) were recorded using the Sedline brain function monitor (Masimo Corporation©, Irvine, California, USA). The sampling frequency of analyzable signal was 179 Hz. The intra operative EEG signal was not collected if the electrode impedance was >5 kΩ. Data collected from standard monitoring included: Pulse Oxygen Saturation (SpO2), Heart Rate (HR), Systolic (SBP), Diastolic (DBP) Blood Pressure and Mean Arterial Pressure (MAP), temperature, Expired CO2 Fraction (EtCO2). Patient demographics were collected during the medical consultation with the anesthesiologist. This study has been approved by the SRLF (Société de réanimation de la langue française) Ethics

Advisory Committee 11–356. To participate, all volunteers had to provide an oral consent before any study-related activities.

Anesthetic Protocol

General anesthesia was induced using total intravenous anesthesia (TIVA) (Absalom and Mason, 2017), with first a standardized administration of opioid agent, then Propofol. For neuroradiology procedures, analgesia was provided using remifentanil while boluses of sufentanil were administered every hour for orthopedic surgery patients. The protocol required a standardized administration for all patients, remifentanil was administered according to the Minto model between 2.5 and 3.5 ng/ml and sufentanil administered in iterative bolus doses between 0.2 and 0.3 μg/kg/hour. An instruction was given to the anesthesiologist in charge to maintain the SEF_{95} within 8 and 13 Hz after the oral tracheal intubation by modulating Propofol TCI. All patients were intubated after muscular relaxation obtained with atracurium besilate injection (0.5 mg/kg). They were mechanically ventilated with a tidal volume of 6–8 ml/kg and a with a respiratory rate adapted to obtain an $EtCO_2$ between 35 and 38 mmHg. The administration of fluids and vasoconstrictors was left to the discretion of the anesthesiologist present in the operating room. The mean arterial pressure was strictly controlled to stay at or above 70 mmHg and temperature controlled above 36°C.

Cognitive Assessment by the Montreal Cognitive Assessment (MoCA)

Cognitive functions were evaluated using the MoCA method 1 day before (D-1) the surgical intervention. We defined the cognitive decline (CD) based on a D-1 MoCA score < 25 points. Patients with a cognitive decline where designate by CD+, CD– otherwise. Two anesthetists and one anesthetist nurse, trained to administer, interpret and score the MoCA tests, carried out all administrations. Prior to each evaluation, we checked for the absence of a significant pain that could affect attention and concentration, for this purpose we used the numerical rating scales and included patients with a score ≤ 4. We also assessed delayed neurocognitive recovery (dNCR) by estimating the drop in MoCA score (ΔMoCA) between the D-1 MoCA (baseline) and follow-up intervention after two days (D+2). A patient was considered with a delayed neurocognitive recovery (dNCR +) if $\Delta\text{MoCA} > 2$ (**Supplemental Material**).

Automatic Alpha Band Power Estimation and Propofol TCI at SEF_{95} 8-13 Hz

The EEG signal was collected from EEG monitor in an .edf format, then processed using Matlab R2018a. The variables were extracted within a 5 min long time window where the spectral edge frequency (SEF) remained in a (Berger et al., 2018) Hz range (stable SEF). We recall that the SEF_{95} corresponds to the frequency below which 95% of the signal total power is found. This index reflects the depth of the general anesthesia, such that an SEF_{95} in 8–13 Hz ensures that patient sedation is appropriate to generate an alpha rhythm. On the contrary, a SEF_{95} outside the stable region might be the sign of burst suppression (deep sedation) or beta activity (light sedation). We used an automatic procedure to detect 5 min long 8–13 Hz SEF_{95} region in the signal (details are provided in the **Supplementary Section**).

Statistical Analysis

Statistical Modeling of Cognitive Decline

We evaluated the risk of presenting a cognitive decline based on age, the combination of Propofol TCI and AP, and all variables considered. Statistical analysis focused on a time window where the SEF_{95} was stable between 8 and 13Hz. Patient CD were labeled using 1 for CD+ (Low MoCA) and 0 otherwise. This binary outcome was modeled as a random variable drawn from a binomial distribution: $CD \sim Binomial\left(1, p\right)$, where using the age we had:

$$logit\left(p\right) = \beta_0 + \beta_1 \cdot Age.\left(AGE\right) \quad (1)$$

Based on TCI and AP, we had:

$$logit\left(p\right) = \beta_0 + \beta_1 \cdot TCI + \beta_2 \cdot AP.\left(HELP\right) \quad (2)$$

For better readability, we have chosen to assign a score from 0 to 100 (%) to the HELP model for each patient reflecting the probability of experiencing cognitive decline.

Finally, to investigate whether or not the three variables together bring complementary information in predicting CD, we extended HELP to include the age such that:

$$logit\left(p\right) = \beta_0 + \beta_1 \cdot TCI + \beta_2 \cdot AP + \beta_3 \cdot Age.\left(HELP2\right) \quad (3)$$

The intercept β_0 as well as coefficients $(\beta_1, \beta_2, \beta_3)$ were optimized numerically based on the data. Models HELP, AGE and HELP2 where compared and evaluated based on the log likelihood. Models were first compared with regard to the data-fit using log likelihood ratio tests. To account for the risk of overfitting, we then compared models based on information criteria penalizing for model complexity in terms of numbers of parameters. We reported the Akaike information criterion (AIC) and Bayesian information criterion (BIC). To explore the data-fit at the level of medical decision-making, we considered receiver operating characteristic (ROC) curves and their areas under the curve (AUC) for each of the three models.

Statistical Inference

Estimating the relative impact of the TCI and AP variables on the HELP score, we computed for each variable, based on the probability from HELP, the marginal effect (ME) $m_{TCI} = \frac{\partial p(CD|TCI,AP)}{\partial TCI}$ and $m_{AP} = \frac{\partial p(CD|TCI,AP)}{\partial AP}$, that we reported by the mean, standard deviation and confidence interval (CI) of the mean (Thomas J. Leeper 2018, margins: Marginal Effects for Model Objects. R package version 0.3.23.). We can remark that TCI (resp. AP) ME for the logistic model HELP is proportional to coefficient β_1 (resp. β_2), thus for instance a null β_1 (null hypothesis) will translate into a null ME ($m_{TCI} = 0$). Finally, we investigated the average ME of one variable conditioned to a specific value of the other one. Such metric assesses the importance of one variable in predicting CD given the value of the second variable. This conditional ME was shown as a graph of the means and standard error.

Statistical Analysis

The description of qualitative data was given in percentage while quantitative ones were reported as median and interquartile range (IQR). Patient characteristics were compared between CD+ and CD– groups using a Mann–Whiney test for quantitative variables and a Chi^2-test for qualitative variables. The significance level for all statistic tests was $\alpha = 0.05$. Statistical analyses were performed using R version 4.0.

RESULTS

Patients

Between November 2018 and May 2019, a total of 56 patients were selected to evaluate the relationship between the per-operative alpha-band, sedation levels during a Propofol based general anesthesia and a pre-operative MoCA psychometric testing. Of these, 14 (25%) did not meet one of the three following conditions: agreed for D-1 MoCA evaluation (4 patients, 7%), had a collected EEG signal (2 missing, 4%) and had a stable SEF_{95} (8 patients, 14%, see **Supplementary Flowchart Figure**). Forty-two patients were therefore analyzed as having a preoperative cognitive assessment and a stable intraoperative EEG period at SEF_{95} 8–13 Hz (74% female, age = 59.4 ± 18.8 yrs.). 40% (17 patients) had CD+ (MoCA pre-operative < 25 points). Fourteen (33%) and 28 (67%) have been admitted for a neuroradiology procedures and orthopedic surgeries, respectively. These patients Propofol TCI and AP were 3.4 [3.0, 3.5] μg/ml and 6.37 ± 3.9 dB, respectively. The median time of surgery was 2.6 h [2.0, 3.3] (**Table 1**).

Difference Between CD+ and CD- Patients

CD+ patients were significantly older (72 ± 12 yrs. vs. 50 ± 17, $p < 0.001$), and had neither significantly more cardiovascular nor neurological risk factors (**Table 1**). Interestingly, CD+ patients had significantly more anti- hypertensive treatments (71 vs. 38%, $p = 0.036$). No significant difference between women and men distribution could be found. The type of surgery was not significantly different between the two groups. Concerning the intra-operative variables, we found that the alpha-band AP was significantly lower in the CD+ group (5.6 ± 2.3 vs. 8.9 ± 3.0 dB, $p < 0.001$). Additionally, these patients required a significantly lower Propofol TCI (3 [2.6, 3.5] μg/ml vs. 3.5 [3, 3.6] μg/ml, $p < 0.01$) to achieve the same anesthetic depth (SEF95: 8–13 Hz). Interestingly, the cumulative time spend in iso-electrical suppression (burst suppression) state was statistically similar between CD + and CD- patients (267 [184; 428] seconds vs. 132 [14; 346] seconds, $p = 0.151$), and was thus poorly associated with CD (AUC = 0.63).

Model Selection to Predict Occurrence of CD

To evaluate AP, TCI, and age for predicting CD, we benchmarked three logistic models based on the age (AGE), TCI+AP (HELP), and the all three variables together (HELP2). The ROC AUC for the AGE was 0.83, then 0.88 for HELP and 0.90 for HELP2 (see **Figure 1A**). We found that compared to AGE, HELP significantly better fitted the data than HELP2 ($p = 0.032$ vs. 0.064, likelihood ratio test). Additionally, the AIC (resp. BIC) dropped from 43.7 (47.25) for AGE to 40.84 (46.05) and 39.42 (46.37) for HELP and HELP2, respectively, suggesting that the HELP model was the best trade-off between prediction score and the number of parameters. In summary, the model HELP, including TCI and AP, better predicted CD than age and was more parsimonious than HELP2. Furthermore, at this stage adding age to TCI and AP did not add obvious information to predict CD. Therefore, we focused on the HELP model. We, hence, extrapolated the probability of CD for TCI ranging from 1 to 7 μg/ml and AP from 1 to 15 dB (see **Figure 1B**). The CD+ and CD- regions are separated by the linear boundary such that a patient is classified as CD + if $TCI < 4 - \frac{1.3}{10} AP$.

Effect of TCI and AP on Probability of CD

We were then interested in describing relative effects the TCI and AP variables had on CD probability using the HELP model. We measured each variable's contribution by reporting average marginal effects. We found that the Propofol TCI had a greater impact on CD probability (ME −0.38 ± 0.24, CI = [−0.45, −0.30], $p = 0.0012$) compared to AP (−0.05 ± 0.03, CI = [−0.06, −0.04], $p = 0.0172$). These findings are coherent since TCI modulates AP, however they also reveal the additional effect of these two variables (see **Figure 2A**). We further analyzed how the average ME of one variable was conditioned by the second. Such analysis aims to explore the impact one specific variable had on the prediction effect of the second one. We found that AP effect on CD probability was small for TCI ranging from 1 to 2 $\mu g/ml$ and from 4 to 5 $\mu g/ml$, however contribution of AP in predicting CD was the most important at a TCI of 3 $\mu g/ml$ (see **Figure 2B**). Such local minimum of conditional ME reveals a range of TCI where AP effect increases up to 44% above the average ME (average: −0.051, CI = [−0.062, −0.041] vs. local min.: −0.074 CI = [−0.081, −0.067]). On the other hand, the Propofol TCI conditional ME remained below −25% per unit of TCI for almost all AP-values and was the most important at AP = 5.5 dB (see **Figure 2C**), showing that weak and strong AP discriminated patients likely to have a CD. In summary, TCI and AP revealed additive and complementary information on CD.

We further proceeded by evaluating the generalization performance of the proposed model of CD (**Figure 3**) to distinct but related data on iso-electrical suppressions (IES), the flat component of the burst-suppression. We considered 56 patients from a previous observational study on the relationship between ABP, TCI and IES (Touchard et al., 2019) in which IES was investigated as a proxy for CD. Results suggest that our proposed model generalizes well-beyond the current dataset and captures physiological variance linked to cognitive decline and drug sensitivity.

DISCUSSION

In the present work, we have found that a lower Propofol requirement (TCI) and a weaker frontal alpha-band power (AP)

TABLE 1 | Characteristic population and difference between CD+ and CD- patients (C.V., cardiovascular).

Variable	All ($n = 42$)	CD– ($n = 25$)	CD+ ($n = 17$)	p
Age (yr)	59.4 ± 18.8	50.48 ± 17.4	72.47 ± 12.2	<0.001
Female	31 (73.8%)	20 (80.0%)	11 (64.7%)	0.268
Orthopedic	28 (67%)	16 (57%)	12 (43%)	0.505
Neuroradiology	14 (33%)	10 (71%)	4 (29%)	
Intervention dura- tion (hours)	2.6[2; 3.3]	2.5[2; 3.1]	3[2; 3.3]	0.533
Comorbidities				
MoCA pre	25 [22; 27]	26.5 [25; 28]	20 [14; 23]	< 0.001
C.V. Risk Factors	27 (65.9%)	14 (58.3%)	13 (76.5%)	0.227
C.V. History	7 (17.1%)	2 (8.3%)	5 (29.4%)	0.077
Cardiovascular treatments	21 (51.22%)	9 (37.50%)	12 (70.6%)	0.036
Reference MAP (mmHg)	100 [90; 105]	100 [83; 105]	100 [93; 105]	0.365
Neuro. History	5 (12.2%)	4 (16.7%)	1 (5.9%)	0.298
Depression (treated)	6 (14.63%)	5 (20.83%)	1 (5.9%)	0.182
Psychiatric treat- ments	8 (19.5%)	6 (25.0%)	2 (11.8%)	0.292
Per-operative				
MAP (mmHg) at SEF95: 8-13 Hz	80 [75; 85]	85 [78; 85]	78 [75; 85]	0.255
T(C°) at SEF95: 8-13 Hz	36.3 [36; 36.6]	36.2 [36; 36.4]	36.4 [36.2; 36.8]	0.423
TCI (μg/ml) at SEF95:8–13 Hz	3.25 [3.0; 3.50]	3.5 [3.0; 3.9]	3 [2.50; 3.0]	<0.001
AP (dB) at SEF95: 8–13 Hz	7.56 ± 3.16	8.88 ± 2.99	5.62 ± 2.34	<0.001
HELP Score (%) at SEF95: 8–13 Hz	35.2 [10.5; 70.4]	10.8 [2.2; 36.9]	71.9 [45.8; 91.5]	<0.001
Cumul. IES (s) (total time)	210 [64; 408]	132 [14; 346]	267 [184; 428]	0.151

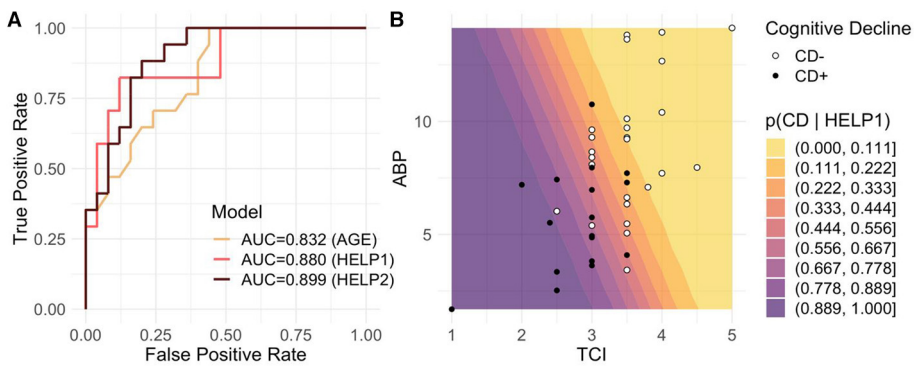

FIGURE 1 | Predicting CD from TCI and AP. We assessed CD prediction based on 3 models including the age (AGE), TCI and AP (HELP1), and the three variables altogether (HELP2). **(A)** We reported logistic based classification results for the three models in a form of ROC curves with their associated AUC, which were 0.832, 0.880, and 0.899 for AGE, HELP1 and HELP2, respectively. Despite better AUC for HELP2, model comparison showed that (HELP2) does not bring significantly more information than HELP ($p = 0.032$ vs. 0.064, likelihood ratio test). We therefore selected HELP1 to model the risk of CD occurrence. **(B)** We investigated the relationship between AP (y-axis), Propofol TCI (a-axis), preoperative MoCA (circles) and the probability to present CD estimated by HELP1 (color gradient). CD+ and CD– patients are depicted by black circles and white circles, respectively. Cold, purple colors indicated high probability to present CD. One can readily see that low MOCA scores were associated with lower TCI and lower alpha power. Some cases are discussed in **Figure 4** concerning patients with MoCA and HELP mismatch [white circles in a cold zone, TCI 2.5 $\mu g/ml$, AP at 6 db (**Figure 4C**) and black circles in a warmer zone, TCI 3 $\mu g/ml$ and alpha band at 10 db (**Figure 4D**)].

observed during a general anesthesia (SEF_{95} in 8–13 Hz) could be used to reveal pre-existing cognitive decline. These two variables performed, in our patient group, better than the chronological age. The effect of AP on the patient probability to have CD was the most prominent at TCI at 3 $\mu g/ml$, and, in a more interesting way, TCI had a much greater effect than alpha band on the likelihood of pre-operative cognitive decline. We further confirmed these finding via HELP model generalization analysis performed on a secondary data set of 56 patients. These results highlight the importance of interpreting cognitive EEG biomarkers (AP) accounting for the hypnotic concentration (TCI). Such information, in daily practice, could help clinician to access patient's biological brain age and then a possible fragility. As more and more studies underline the influence of

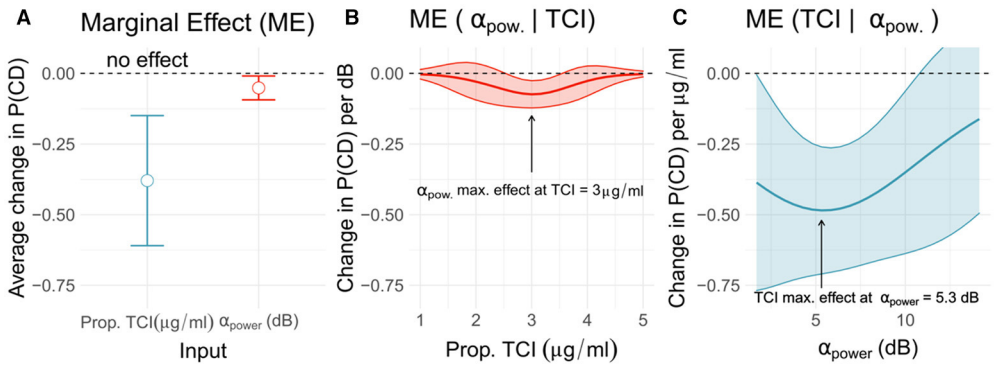

FIGURE 2 | Marginal effects of TCI and alpha power of CD probability. The present triptych shows effect of TCI and AP on the patient's probability to be CD, estimated from the HELP model. On the panel **(A)**, marginal effect means (circle) and standard deviation bar errors are reported for TCI (red) and AP (blue). The two variables contribute to predict the onset of CD among patients, although TCI appears to more strongly affect CD probability compared to AP. Panels **(B,C)** show conditional marginal effects reflecting change in one variable effect relatively to the other variable values. The AP effects is the most important at a TCI of 3 $\mu g/ml$ **(B)**, while TCI strongly impact prediction for an AP of 5.5 dB. Consequently, effect of AP in predicting CD is not homogeneous as TCI changes, with a maximal effect near 3 $\mu g/ml$.

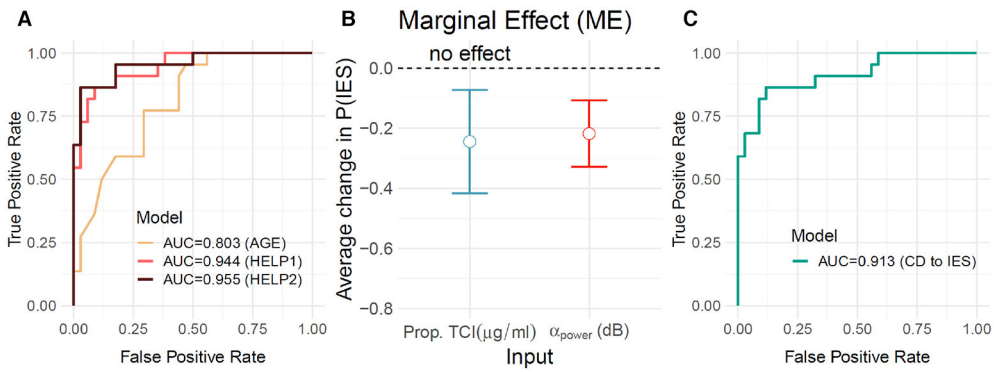

FIGURE 3 | Generalization testing on burst suppression data. To probe the generalization of the proposed model beyond the given sample we considered the observational data from Touchard et al. (2019) in which burst suppression was studied as a proxy for cognitive decline. The dataset comprised 56 patients sedated with Propofol. None of these patients was included in the previous analyses. In a first step **(A)**, we applied the models as defined in the previous analyses (**Figures 1**, **2**) to predict the occurrence of IES during the induction period. HELP1 (TCI + ABP) predicted IES (AUC=0.94, orange) better than AGE (AUC=0.80, yellow). Prediction did not increase significantly for HELP2 (TCI + ABP + Age) as compared to HELP1. We then inspected the HELP1 (IES) model **(B)**. As in previous analyses, TCI (blue) and ABP (red) showed complementary average marginal effects (mean±SE: −0.25±0.09, −0.22±0.06 for TCI and ABP, respectively). We then directly quantified generalization performance **(C)** of the HELP1 (CD) on the IES dataset (AUC=0.91). The results suggest that the proposed HELP model generalizes beyond the observed sample and captures cognitive and physiological factors related to postoperative outcome.

the preoperative cognitive state on the post-operative outcome (Brown et al., 2016; Culley et al., 2017). Taking advantage of general anesthesia to evaluate brain responses to a stress and identify a given vulnerability seems to be an opportunity to be seized, especially as growing number of anesthesia are performed every year and mostly concerns middle age and elderly patients.

Patients with higher preoperative MoCA scores required on average a higher TCI to obtain stable intra-operative EEG-signals within an 8–13 Hz SEF_{95}. These findings are consistent with Laalou and colleagues who reported that TCI was directly influenced by pre-operative cognitive performances (Laalou et al., 2010). In our cohort, the Propofol requirement represented by the TCI set to achieve a SEF_{95} between 8 and 13 Hz has a major effect on the probability of experiencing cognitive decline. When TCI is either very low or high, it is alone sufficient to classify whether the patient is at risk or not. All of our patients with required TCI below 2.5 $\mu g/ml$ had preoperative cognitive decline and those requiring more than 4 $\mu g/ml$ of Propofol had a good cognitive health. Despite AP playing a minor role in predicting CD in this situation (**Figure 2B**), patients with low TCI had nonetheless a low AP too (**Figure 1B**).

The association between low alpha-band and cognitive impairment are consistent with recent work from Koch and colleagues (Koch et al., 2019). The analysis of the average AP effect (average marginal effect **Figure 2A**) showed a significant association with CD. In our study, conditional analysis exposed an inverse U-shaped behavior where AP plays a more important role in predicting CD when the TCI values range from 2.5 to 4 $\mu g/ml$ with maximal impact at TCI 3 $\mu g/ml$. Within such range, it is in general not clear for the practitioner whether the patient

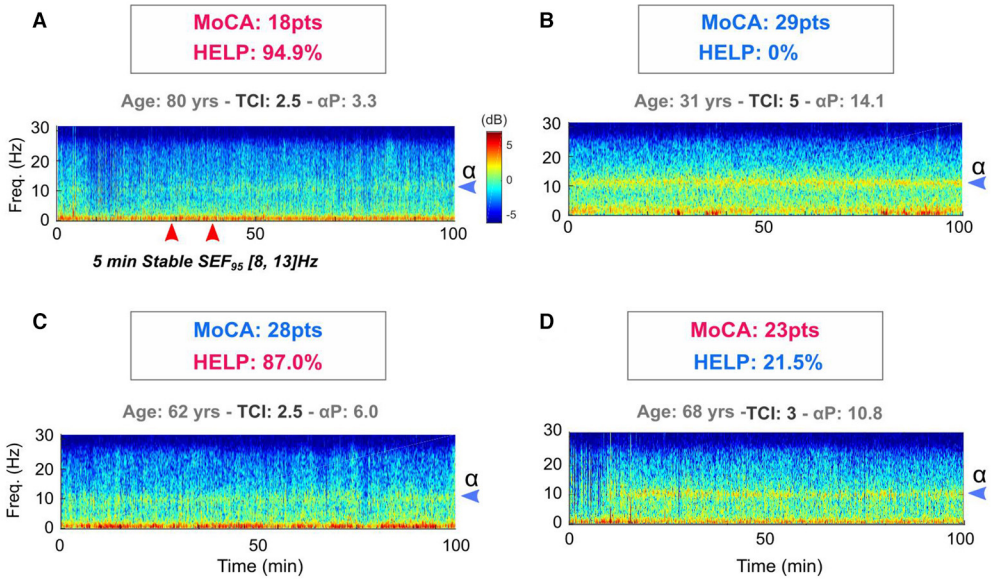

FIGURE 4 | The potential contribution of intra operative HELP model in relation to preoperative MoCA in the detection of cognitive decline of anesthetized patients. For two subjects **(A,B)**, there are classification concordance between MoCA and HELP model. These are deliberately caricatural situations in order to clearly differentiate the intraoperative characteristics that exist between a subject with a low MoCA **(A)** and young subject without cognitive impairment **(B)**. In these situations, neither the HELP model, nor the pre-operative cognitive evaluation provides information in relation to the date of birth. An 80-year-old subject **(A)** is at risk of cognitive complications and a 32-year-old patient **(B)** is not. Things could be more interesting in MoCA and HELP mismatch situations for middle-aged patients **(C,D)**. If subject C presents a normal pre- operative MoCA and is relatively young, it is striking to observe his weak peroperative variables. In fact, this 62-year-old patient suffered a ruptured aneurysm at age 42 with a major subarachnoid hemorrhage that warranted a lengthy intensive care unit hospitalization and described memory and attention complaints later in life. HELP model could appear more robust to detect past and so maybe future brain suffering. On the contrary, the patient D (68 years old woman) presents a robust HELP score derived from the model, discordant with a low preoperative MoCA. The clinical history of this patient with difficult socioeconomic conditions shows chronic anxiety disorders and a marked apprehension of the upcoming surgery. Considering these two situations, the intraoperative variable could be used to help stratify perioperative cognitive risk when MoCA is taken in default, particularly for middle-aged patients (50 to 70 years old).

is fragile or not, which may explain why alpha power helps to further distinguish CD from non-CD patients.

In summary, once a SEF_{95}-defined stable anesthesia is achieved, the associated TCI is collected. If the TCI is high ($>$ 4 $\mu g/ml$) or low ($<$ 2.5 $\mu g/ml$), then it is sufficient to stratify the risk, otherwise we have to evaluate the alpha-band power. There, the lower the alpha-band power, the greater the probability of cognitive decline. Nevertheless, one should keep in mind that AP is influenced by TCI (Purdon et al., 2015; Cartailler et al., 2019). Indeed, a patient with good cognitive performance might have a low AP in the case where the TCI would have been high enough. In the light of our results, establishing relationships between AP, age and cognition without considering the Propofol concentration could exposes one to a lack of precision at best and to risk stratification errors at worst.

Despite important effect sizes, the number of patients was small. We attempted to address this issue by generalizing the HELP model to the dataset from our previous study (Touchard et al., 2019) and found that using iso-electrical suppression was linked to cognitive fragility. Still, as explained in detail by Wildes and colleagues, burst-suppression interpretation is protocol-dependent, hence might not always be the best indicator of CD. This is perfectly well illustrated by the poor association we found between CD and IES in the new set of 42 patients for whom IES was proactively avoided. Another limitation concerns the population-based pharmacokinetics estimation of TCI within the TIVA framework. These models could be ill estimated for frail patients, especially as TIVA models, such as Schnider's, do not adapt for either variations in cardiac output or clearance that often occur among fragile patients. Therefore, in such situation, TCI values predicted by the model would not be true and hence might fail to reflect a specific cognitive fragility.

The MoCA psychometric test also possesses intrinsic limitations although it is a fair trade-off between a short test and one evaluating several cognitive functions. It is particularly interesting in detecting mild cognitive impairments which are considered to be pre-clinical signs of Alzheimer dementia (Nasreddine et al., 2005). However, the trained human resources required to administer MoCA is a limiting condition often preventing its routine use in perioperative period.

Additionally, false positive CD diagnosis might occur for several reasons including stress or pain (**Figure 4**). On the contrary, patients with significant cognitive reserve might pass the neuropsychological screening, yet still, show a cognitive fragility that could manifest itself as a post-operative cognitive dysfunction, such as delirium. For these reasons, EEG and hypnotic concentration are physiological and biological measures that could be more appropriate to detect patients at risk of developing neurocognitive disorders.

CONCLUSION

The power of the alpha band combined with the concentration of Propofol required to achieve unconsciousness could be an effective, objective and reliable tool to detect cognitive decline in the preoperative period. This approach promises to minimize human bias using intra-operative data already collected in routine care. Future studies will need to be conducted to test this type of indirect cognitive assessment by intra operative data modeling (HELP model) for predicting post-operative chronic cognitive disorder by comparing or implementing it to preoperative neuro-cognitive scales. This approach might allow anesthesiologists to play an active role in detecting, predicting and preventing neurocognitive disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the SRLF (Société de réanimation de la langue française) Ethics Advisory Committee 11–356. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CT and JC: study design, data analysis, patient recruitment, data collection, and redaction of the manuscript. CL and JS: patient recruitment and data collection. DS: data analysis. EM: patient recruitment and data collection. JJ and JM: data collection. EG: data analysis. DE: data analysis and manuscript redaction. FV: study design, patient recruitment, data collection, and manuscript redaction. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

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Individual Differences in Interoceptive Accuracy Are Correlated With Salience Network Connectivity in Older Adults

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Interoceptive accuracy refers to the ability to consciously perceive the physical condition of the inner body, including one's heartbeat. In younger adults, interoceptive accuracy is correlated with insular and orbitofrontal cortical connectivity within the salience network (SN). As interoceptive accuracy and insular cortex volume are known to decrease with aging, we aimed to evaluate the correlation between SN connectivity and interoceptive accuracy in older adults. 27 older adults (mean age, 77.29 years, SD = 6.24; 19 female) underwent resting-state functional magnetic resonance imaging, followed by a heartbeat counting task and neuropsychological test. We evaluated the correlation between interoceptive accuracy and SN connectivity with age, sex, cognitive function, and total gray matter volume as covariates. Region of interest-to-region of interest analyses showed that interoceptive accuracy was positively correlated with the functional connectivity (FC) of the left rostral prefrontal cortex with the right insular, right orbitofrontal, and anterior cingulate cortices [$F(6,16) = 4.52$, false discovery rate (FDR)-corrected $p < 0.05$]. Moreover, interoceptive accuracy was negatively correlated to the FC of the left anterior insular cortex with right intra-calcarine and visual medial cortices ($F(6,16) = 2.04$, FDR-corrected $p < 0.10$). These findings suggest that coordination between systems, with a positive correlation between left rostral prefrontal cortex and the SN and a negative correlation between left insular cortex and vision-related exteroceptive brain regions, is important for maintaining interoceptive accuracy in older adults.

Keywords: interoception, insular cortex, salience network, heartbeat counting task, posterior–anterior shift in aging

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INTRODUCTION

Interoception collectively refers to afferent sensory information arising from the sensation, perception, and awareness of afferent feedback from the viscera, which maintains homeostatic function (Craig, 2002). Interoception is distinguishable from exteroception and proprioception (Sherrington, 1948). Renewed interest in interoception parallels a growing appreciation that

cognition is embodied, and that cognitive and emotional processes are biased by extracerebral changes, as captured; for example, in the somatic marker hypothesis (Damasio et al., 1991). Furthermore, interoceptive ability is relevant to psychological construction models of emotion that propose a basis for emotions as the emergent products of psychological ingredients, which are some form of information from the body (primary) and a process by which internal sensory or affective states (secondary) are made meaningful as related to or caused by the external surroundings (Gross and Barrett, 2011).

Heartbeat perception is frequently used in the measurement of interoceptive awareness, as it is simple, practical, and less invasive than other measures. Previous task-related functional magnetic resonance imaging (fMRI) studies have revealed that interoceptive accuracy is correlated with activity in the insular cortex and anterior cingulate cortex (ACC), regardless of the interoceptive task used (Critchley et al., 2004; Schulz, 2016; Stern et al., 2017). Furthermore, resting state fMRI studies, which use the temporal correlation between fluctuations in different areas as a measure of intrinsic functional connectivity (FC), have identified the salience network (SN), involving the bilateral anterior insular cortices and dorsal ACC, as well as the anterior prefrontal cortex (aPFC), supramarginal gyrus (SMG), striatum/basal ganglion, thalamus, and cerebellum, as important for integrating highly processed sensory data with visceral, autonomic, and hedonic markers, to guide the appropriate behavior (Seeley et al., 2007; Guo et al., 2012). Chong et al. (2017) examined the relationship between SN connectivity and interoceptive accuracy in 26 healthy young adults and found a positive correlation between heartbeat counting accuracy and salience network functional connectivity (SN FC) of the right and left posterior insular cortices, but no correlation was found for the FC of the ACC.

Basic processes in numerous sensory modalities, such as exteroception and proprioception, decrease with aging. Numerous studies have shown age-related decreases in primary somatosensory cortex (Good et al., 2001; Sowell et al., 2003; Salat et al., 2004), insular cortex (Good et al., 2001; Resnick et al., 2003), and SN connectivity (Onoda et al., 2012). However, only a few studies have examined the relationship between age and interoception. Although several studies (Khalsa et al., 2009b; Murphy et al., 2017) have reported that age is negatively correlated with interoceptive accuracy, to our knowledge, no study has examined the relationship between SN connectivity and interoceptive accuracy in older adults. Therefore, the current study sought to fill this knowledge gap by examining the correlations between SN connectivity and individual differences in interoceptive accuracy, as assessed by the heartbeat counting task, in a group of older adults. We used the heartbeat counting task, rather than the heartbeat discrimination task, since it is more reflective of the internal monitoring processes (Critchley et al., 2004; Pollatos et al., 2005; Chong et al., 2017). We hypothesized that better performance on the heartbeat counting task would be correlated with greater FC of the SN.

MATERIALS AND METHODS

Participants

Thirty-one older adults (mean age = 77.08, standard deviation SD = 1.19, range = 61.2–89.0; 22 female) were enrolled in the study. However, we excluded the data of four older adults because of a failure to follow the instructions or missing values. Thus, 27 older adults (mean age = 77.30 years, SD = 6.20, range = 61.20–86.90; 19 female) were finally included. 26 participants were right-handed and 1 participant was left-handed. Classification of handedness was based on the modified 25-item version of the Edinburgh Inventory (Oldfield, 1971). In this study, the sample size could not be calculated from the effect size, power, and significance level because of the nature of the MRI imaging analysis (the statistical analysis would be performed on numerous voxels with multiple comparison correction). Therefore, the sample size was determined with reference to Chong et al. (2017).

Participants comprised outpatients of the Center for the Diagnosis of Dementia, Kyoto Prefectural University of Medicine, as well as volunteers who lived locally or were a member of an employment service center for the elderly, who were aged 60 years or more and had maintained activities of daily living. Participants recruited from the Center for the Diagnosis of Dementia included both healthy adults and those with mild cognitive impairment, based on the results of various neuropsychological tests. The exclusion criteria were as follows: dementia or intellectual disability; a history of mental illness; brain injury; drug or alcohol abuse; serious impairment in vision, hearing, or the function of both hands; and unable to undergo MRI scanning. This study was conducted from October 28, 2017 to September 28, 2019, and was approved by the ethics committees of Kyoto Prefectural University of Medicine (ERB-C-853-3). Informed consent for participation was obtained from all participants.

Procedures

After completing the informed consent process, participants were taken to the MRI scanner at the Kajiicho Medical Imaging Center, where they completed a neuroimaging session (see section "Imaging Acquisition Protocol"). Participants were then escorted back to the university hospital to complete the heartbeat counting task and the Mini-Mental State Examination (MMSE).

Imaging Acquisition Protocol

The participants underwent one neuroimaging session on a 3T Philips Achieva 3.0 Quasar Dual (Royal Philips, Japan) with a 32-channel head coil. The session comprised a high-resolution T1-weighted three-dimensional magnetization-prepared rapid gradient-echo (3D MPRAGE) at 3.0 T scan of the entire brain in 170 sagittal slices (magnetization prepared rapid gradient echo sequence, with repetition time and echo time = shortest, flip angle = 9°, field of view = 256 × 256 mm^2, and voxel size = 1.0 × 1.0 × 1.2 mm, slice thickness = 1.2 mm) and a 10-min resting-state fMRI scan (T2×-weighted echo planar sequence, with repetition time = 2500 ms, echo time = 30 ms, flip angle = 80°, 40 axial slices, field of view = 212 × 212 mm^2, voxel

size = 3.31 × 3.31 × 3.20 mm, matrix size = 64, no interslice skip, slice thickness = 3.2 mm, 0.8-mm gap, and interleaved collection), during which participants were instructed to remain awake and fixate on a black cross in the center of a white screen.

Heartbeat Counting Task

All participants completed a heartbeat counting task immediately after scanning (i.e., on the same day). In accordance with previous work (Schandry, 1981; Pollatos et al., 2005; Meissner and Wittmann, 2011; Kuehn et al., 2016), participants were instructed to attend to their own heartbeat and silently count the number of beats within five intervals (15, 25, 35, 45, and 100 s; one trial each). Since older adults generally have decreased interoceptive accuracy, we included a 15-s interval, allowing us to measure interoceptive accuracy in short to long intervals. The start and end of each counting interval were indicated by the experimenter. At the end of each interval, participants were prompted to indicate the number of heartbeats counted, followed by a 10-s rest period before the start of the next counting interval. Additionally, participants were instructed to refrain from opening their eyes or using any other physical strategies to aid in the counting of heartbeats. Moreover, to confirm a distinction between heartbeat and time counting, participants were instructed to count time silently over the same five intervals (15, 25, 35, 45, and 100 s), immediately after completing all heartbeat counting intervals.

The heartbeats of the participants were continuously monitored throughout the heartbeat counting task using a pulse oximeter (model 9560 Onyx 2, Star Product Ltd., Tokyo, Japan). While it is possible that different results would be observed with a different measure of interoception allowing easier detection of heartbeats, almost all existing studies of the relationship between interoception and aging have employed this task as an objective index of interoceptive accuracy. Using a finger clip pulse oximeter to measure their own pulse is easier than using an electrocardiogram (ECG) pulse oximeter and a hard-clip (but not soft-clip) oximeter, and the ECG pulse oximeter is correlated with increased perception of heartbeat at the finger (Murphy et al., 2019). The average pulse rate was sampled, and participants were given a 5-min rest to stabilize their pulse rate before the task was started.

Interoceptive accuracy scores were calculated using the formula in Pollatos et al. (2005) and Chong et al. (2017); however, we calculated relative differences, rather than absolute differences, due to remaining individual differences in the number of counted heartbeats compared to the recorded number of heartbeats in older adults who overestimated or underestimated the number of heartbeats in a given time interval. For each participant, accuracy in the perception of the heartbeat was quantified by first taking the relative difference between the actual number of heartbeats recorded by the pulse oximeter and the number of heartbeats counted by the participant, divided by the actual number of heartbeats. This value was then subtracted from 1 and averaged across all trials to yield a heartbeat counting score, such that scores near 100 indicate higher accuracy. Moreover, scores less than 100 indicate that the number of counted heartbeats was underestimated (relative to the number of recorded heartbeats). The mathematical formula was, thus, as follows:

$$\frac{1}{5}\Sigma\left(1-\frac{\text{recorded heartbeats} - \text{counted heartbeats}}{\text{recorded heartbeats}}\right)\times 100$$

Time accuracy scores were calculated as the relative difference between the actual time and the time counted. This value was also subtracted from 1 and averaged across all trials to yield a time counting score, such that scores near 100 indicate higher accuracy in time counting. The mathematical formula was, thus, as follows:

$$\frac{1}{5}\Sigma\left(1-\frac{\text{actual time} - \text{counted time}}{\text{actual time}}\right)\times 100$$

MMSE

The MMSE (Folstein et al., 1975) was used to evaluate cognitive function and exclude patients with dementia. The MMSE is a widely used neuropsychological battery to assess cognitive function and screen for dementia. The components of the MMSE are as follows: (1) orientation, (2) immediate memory, (3) attention and calculation, (4) delayed memory, and (5) language. The total score of the MMSE is 30 points, and the mean score is 27.6 (SD = 1.7) in healthy older adults.

Image Preprocessing

Structural and functional images were preprocessed, using CONN's default preprocessing pipeline, in CONN Toolbox version 18.b (Whitfield-Gabrieli and Nieto-Castanon, 2012), implemented in the MATLAB R2016b environment (The MathWorks, Inc., Natick, MA, United States). After the first ten volumes were discarded to allow for magnetic field stabilization, functional scans underwent cerebrospinal fluid (CSF)/white matter (WM) noise removal via the anatomical component-based noise correction procedure (aCompCor), slice time- and motion-correction (realigned), scrubbing, unwarping, co-registration to structural scans, linear detrending but no despiking, normalization to the Montreal Neurological Institute (MNI) atlas space, and spatial smoothing with an 8-mm full width at half maximum (FWHM) Gaussian kernel.

Subject-level gray matter (GM) volume probability maps were derived from T1-weighted images using voxel-based morphometry (VBM). VBM was performed using Statistical Parametric Mapping (SPM12) (Wellcome Trust Centre for Neuroimaging)[1], and included segmenting individual T1-weighted images into GM volume, WM and CSF using an adaptive Maximum A Posterior technique (Rajapakse et al., 1997), which eliminates the use of tissue priors.

Statistical Analysis

Functional connectivity analyses were performed using the default FC processing pipeline in the CONN Toolbox version 18.b (Whitfield-Gabrieli and Nieto-Castanon, 2012). In this processing pipeline, using a component-based noise correction method (Behzadi et al., 2007), physiological and other spurious sources of noise were removed together with the movement-

[1]http://www.fil.ion.ucl.ac.uk/spm/software/spm12/

and artifact-related covariates. The blood oxygen level dependent (BOLD) signal from the cerebral white matter and ventricles was removed prior to seed-based connectivity analysis using a principal component analysis of the multivariate BOLD signal within each of the masks obtained from the segmented T1-weighted scan (Woodward et al., 2011). The residual BOLD time-series was then band-pass filtered (0.008–0.09 Hz). We generated seed region of interest (ROI) to target ROI connectivity maps for each participant using reproducibly demonstrated SN seeds. The seed ROIs comprised 10-mm diameter spheres at the following anatomical locations and spatial coordinates: bilateral rostral prefrontal cortex (Brodmann's area (BA) = 10; right, $x = 32$, $y = 46$, $z = 27$; left, $x = -32$, $y = 45$, $z = 27$), ACC (BA = 32, $x = 0$, $y = 22$, $z = 35$), bilateral anterior insular cortex (BA = 13; right, $x = 47$, $y = 14$, $z = 0$; left, $x = -44$, $y = 13$, $z = 1$), and bilateral SMG (BA = 40; right, $x = 62$, $y = -35$, $z = 32$; left, $x = -60$, $y = -39$, $z = 31$). The target ROIs comprised a whole-brain set of 164 ROIs defining the BA (Talairach atlas; Lancaster et al., 2000). The seed ROIs are provided by the CONN toolbox, and represent core topological nodes within the SN. The reasoning for the identification and use of these seeds is described in greater detail by the originators of the CONN toolbox (Whitfield-Gabrieli et al., 2011).

In a group-level analysis, one-sample general linear model analyses were conducted to examine the positive and negative correlations between heartbeat counting accuracy and SN FC, with age, sex, MMSE score, and total GM volume as covariates. A whole-brain height threshold of $p < 0.001$ (uncorrected) was used to identify areas with a significant correlation. A false discovery rate (FDR)-corrected threshold of $p < 0.05$ at this height threshold was applied for all reported clusters.

Group-level independent component analysis (ICA), using the CONN Toolbox version 18.b, was conducted to identify the network of functionally connected brain regions during resting state that may be correlated with interoceptive accuracy scores. This involved the application of the fast ICA algorithm to volumes concatenated across subject and resting state conditions to identify independent spatial components (ICs) and the back-projection of these components to individual subjects, which produced regression coefficients maps representing connectivity between the network and every voxel in the brain. Forty ICs were identified using spatial overlap of suprathreshold areas (Dice coefficient; Rombouts et al., 1998), based on CONN's default network atlas with ROIs characterizing an extended set of salience (7 ROIs), visual (4 ROIs) network. We selected 40 ICs based on previous research suggesting that ICA results are only affected by the number of ICs when it is smaller than the number of source signals (Ma et al., 2007), in addition to coverage of most signal variance. Forty ICs were included in multiple regressions with interoceptive accuracy scores. For each network, the resulting statistical maps had a voxel threshold of $p < 0.05$ (uncorrected) and cluster threshold of $p < 0.05$ (FDR-corrected). All coordinates reported below refer to peak activations in anatomical MNI space.

In comparison to a previous study in younger adults (Chong et al., 2017), to elucidate which insular subdivision(s) and their connected brain regions are linked to interoceptive accuracy, we further examined the relationship between voxel-wise connectivity maps of each insular subdivision and the heartbeat counting accuracy scores. We first derived eight insular subdivision seeds (both lateral mid-posterior, anterior-ventral, anterior-dorsal, and middle insular) from Kurth et al. (2010). The CONN Toolbox version 18.b performs seed-based analysis by computing the temporal correlation between the BOLD signals from a given seed to all other voxels in the brain. The residual BOLD time-series was then band-pass filtered (0.008–0.09 Hz). A linear regression analysis was conducted to remove signals from the ventricular area and white matter. To minimize the effects of head movement, motion parameters were included in the linear regression analysis. To estimate the strength of an FC, correlation coefficients were computed and converted to z-values using Fisher's r-to-z transformation. Finally, whole-brain voxel-wise regression of each seed-based connectivity map against the heartbeat counting accuracy scores was conducted to identify brain regions connected to the insular subdivisions correlated with heartbeat counting accuracy scores. A whole-brain height threshold of $p < 0.05$ (uncorrected) was used to identify areas with a significant correlation. A FDR-corrected threshold of $p < 0.05$ at this height threshold was applied for all reported clusters.

Spearman's rank correlation coefficient (ρ) was used to examine correlations among age, sex (with 0 indicating female and 1 indicating male), total MMSE score, heartbeat counting accuracy scores, time counting accuracy scores, and GM volume of set of salience (7 ROIs) and visual network (4 ROIs). Because we cannot hardly say with any finality that sample size is enough for following normal distribution, we calculated Spearman's rank correlation coefficients (ρ). Moreover, we adjusted the p-value using Benjamini and Hochberg's method (Benjamini and Hochberg, 1995). Data were analyzed using SPSS 25 (IBM Corp., Armonk, NY, United States), with $p < 0.05$ considered to be significant.

RESULTS

Behavioral Results

The mean age, sex, total MMSE score, and accuracy scores (for heartbeat and time counting) are listed in **Table 1**. The heartbeat counting accuracy score ranged between 37.64 and 101.37 (78.96 ± 18.09). Furthermore, the heartbeat counting

TABLE 1 | Characteristics for each variable.

	Mean	SD	Min	Max
Age	77.30	6.20	61.20	86.90
Sex	19 female		–	–
MMSE	27.96	6.24	22.0	30.0
IA	77.96	18.09	37.64	101.37
TA	103.14	23.49	54.79	156.94

MMSE, Mini-Mental State Examination; IA, Interoceptive Accuracy; TA, Time counting Accuracy.

accuracy score was not significantly correlated with age, sex, MMSE score, or time counting accuracy score (**Table 2**).

Association Between SN FC and Heartbeat Counting Accuracy

The results of the ROI-to-ROI analysis are illustrated in **Figure 1**. We found a significant positive correlation between the heartbeat counting accuracy scores and the FC of the left rostral prefrontal cortical seed with the group-level SN ($F(6,16) = 4.52$, FDR-corrected $p < 0.05$). Moreover, higher heartbeat counting accuracy was associated with increased FC of the left rostral prefrontal cortical seed with the right insular cortex ($t(21) = 4.86$, FDR-corrected $p < 0.05$; **Figure 2A**), right orbitofrontal cortex ($t(21) = 4.44$, FDR-corrected $p < 0.05$; **Figure 2B**), and ACC ($t(21) = 3.89$, FDR-corrected $p < 0.05$; **Figure 2C**), even when controlling the effects of age, sex, MMSE score, and GM volume. Additionally, we found a significant negative correlation between heartbeat counting accuracy and the FC of the left anterior insular cortical seed with the group-averaged SN ($F(6,16) = 2.04$, FDR-corrected $p < 0.10$). Moreover, higher heartbeat counting accuracy was associated with decreased FC of the left anterior insular cortical seed with right intra-calcarine cortices ($t(21) = -4.20$, FDR-corrected $p < 0.05$; **Figure 2D**), and visual medial cortex ($t(21) = -4.04$, FDR-corrected $p < 0.05$; **Figure 2E**), even after controlling the effects of age, sex, MMSE score, and total GM volume. Scatterplots depicting the relationship between heartbeat counting accuracy and FC values are shown in **Figure 2**.

The results of the ICA analysis are illustrated in **Figure 3**. We found a significant positive correlation between the heartbeat counting accuracy scores and the cerebellum (peak voxel = 56, −66, −36; cluster size of 1135 voxels, $t(21) = 4.56$ FDR-corrected $p < 0.05$). Additionally, we found a significant negative correlation between the heartbeat counting accuracy scores and both the lateral orbitofrontal cortex (peak voxel = 2, 18, −20; cluster size of 1231 voxels, $t(21) = -4.79$, FDR-corrected $p < 0.05$) and caudate nucleus (peak voxel = 12, 14, −1; cluster size of 1135 voxels, $t(21) = -3.90$ FDR-corrected $p < 0.05$), even after controlling the effects of age, sex, MMSE score, and GM volume. Moreover, no significant correlations were observed between visual network connectivity and heartbeat counting accuracy scores, or between GM volumes of a set of SN (7 ROIs) and heartbeat counting accuracy scores.

We conducted additional analysis on the correlation between four subdivision(s) of the insular cortex and heartbeat counting accuracy scores. Higher heartbeat counting accuracy was associated with decreased FC of the left middle insular gyrus as seed with the visual medial cortex ($t(21) = -4.53$, FDR-corrected $p < 0.05$), left lingual gyrus ($t(21) = -4.15$, FDR-corrected $p < 0.05$), and right supracalcarine cortex ($t(21) = -3.91$, FDR-corrected $p < 0.05$), even after controlling the effects of age, sex, MMSE score, and total GM volume.

DISCUSSION

In the present study, we performed an ROI-to-ROI analysis to investigate the relationships between interoceptive accuracy and SN connectivity. Interoceptive accuracy, as assessed by the heartbeat counting task, was positively associated with FC between the left rostral prefrontal cortex and brain regions within the SN, including the right insular cortex, ACC, and right orbitofrontal cortex. In contrast, interoceptive accuracy was negatively associated with FC between the left anterior insular cortex and visual-processing brain regions, including the right intra-calcarine cortices and visual medial cortex. Moreover, ICA analysis showed that interoceptive accuracy was positively associated to cerebellum and negatively associated with both lateral orbitofrontal cortex and caudate nucleus. Therefore, this study revealed that the SN plays an important role in maintaining interoceptive accuracy in older adults.

Neural Correlates of Interoception

Neural signals for interoception are generated by the transmission of information regarding the physical condition to the brain via the afferent system, with control by the efferent system. The autonomic nervous system, consisting of the sympathetic, parasympathetic, and enteric nervous systems, and visceral afferents, is greatly involved in interoception. Autonomic nervous system output involves several interconnected areas distributed throughout the forebrain and brainstem. The primary forebrain autonomic areas are the insular cortex, ACC, and midcingulate cortex. Especially, the posterior insular cortex receives and integrates interoceptive or bodily sensations. The posterior insula cortex projects to the anterior insula, which integrates interoceptive signals and is involved in the conscious experience of bodily sensation, including the timing of the heartbeat. The subgenual ACC projects to the autonomic areas involved in parasympathetic control of the heart. These visceromotor cortices play a major role in interoception by issuing prediction signals on the expected state of the body

TABLE 2 | Spearman's ρ correlation coefficients among variables.

	Age	Sex	MMSE	IA	TA
age	–	−0.12 (−0.48 ≦ ρ ≦0.27)	−0.11 (−0.47 ≦ ρ ≦0.28)	0.03 (−0.35 ≦ ρ ≦0.41)	0.03 (−0.35 ≦ ρ ≦0.41)
sex		–	−0.32 (−0.62 ≦ ρ ≦0.07)	0.46 (0.10 ≦ ρ ≦0.71)	0.10 (−0.29 ≦ ρ ≦0.46)
MMSE			–	−0.06 (−0.43 ≦ ρ ≦0.33)	0.09 (−0.30 ≦ ρ ≦0.45)
IA				–	−0.08 (−0.45 ≦ ρ ≦0.31)
TA					–

MMSE, Mini-Mental State Examination; IA, Interoceptive Accuracy; TA, Time counting Accuracy, 95% confidence intervals in parentheses.

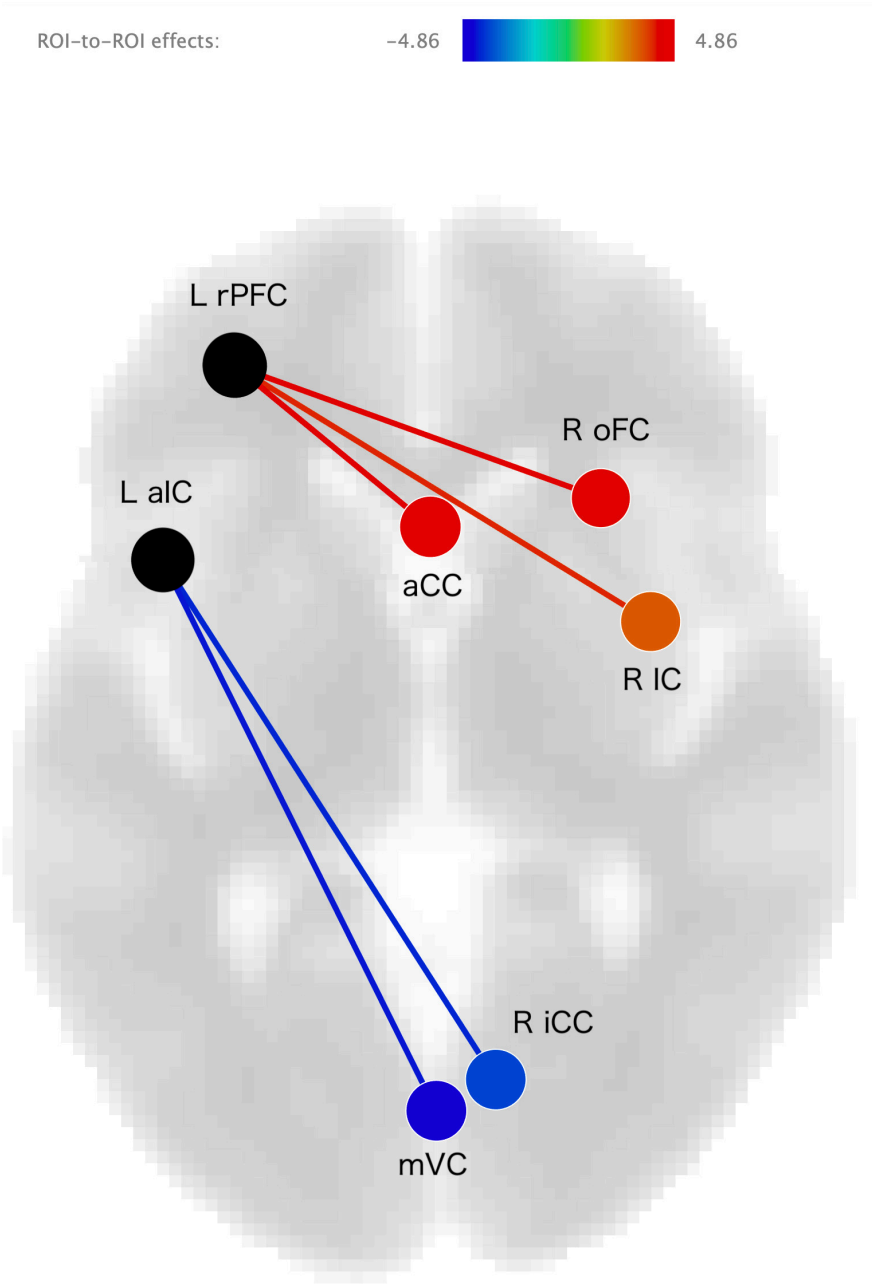

FIGURE 1 | Visual depiction of significant positive correlations (red) and negative correlations (blue) between seed functional connectivity and interoceptive accuracy scores (after statistically accounting for age, sex, MMSE score, and GM volume). Seed regions are labeled in black. L rPFC, left rostral prefrontal cortex; R IC, right insular cortex; R oFC, right orbitofrontal cortex; aCC, anterior cingulate cortex; L aIC, left anterior insular cotex; R iCC, right inter-calcarine cortex; mVC, medial visual cortex; MMSE, Mini-Mental State Examination; GM, gray matter.

based on previous experience; these interoceptive predictions are then compared to current visceral sensations as a mechanism to correct autonomic output (Bennarroch, 2020). In particular, the transmission of information from the right insular cortex to the ACC and orbitofrontal cortex has been observed in young adolescents (Dennis et al., 2014). Furthermore, the report by Craig (2009) hypothesized that subjective perceptions of internal sensations depend on the right anterior insular cortex.

In an fMRI study, Critchley et al. (2004) investigated the neural circuitry underlying performance on a heartbeat discrimination task; the results revealed the right anterior insular cortex as an important region for interoceptive accuracy. Moreover, GM volume in the right anterior insula, orbitofrontal cortex, and midline cerebellum was correlated with interoceptive accuracy. Schulz (2016) conducted a meta-analysis of nine studies and found that heart-focused interoceptive accuracy, and

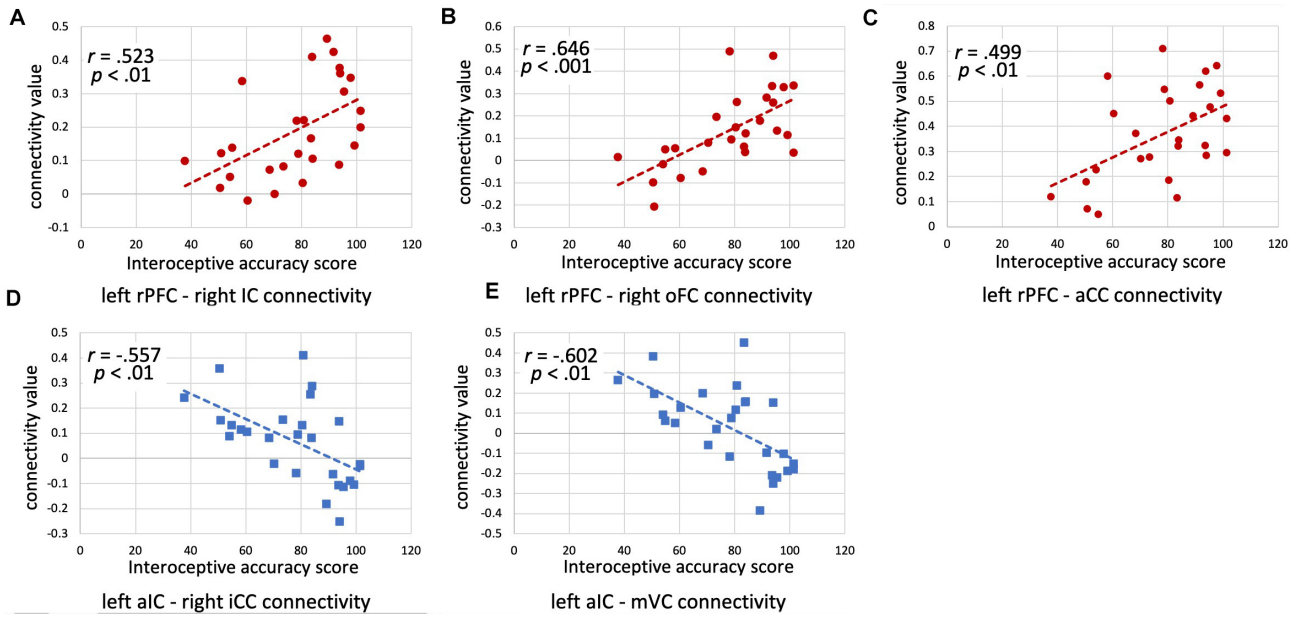

FIGURE 2 | Scatterplots illustrating the correlations between interoceptive accuracy scores and functional connectivity values (z-scores) for each ROI seed-target pair (accounting for age, sex, and MMSE score) are shown. **(A)** Left rPFC–Right IC connectivity; **(B)** Left rPFC–Right oFC connectivity; **(C)** Left rPFC–aCC connectivity; **(D)** Left aIC–Right iCC connectivity; **(E)** Left aIC–mVC connectivity. rPFC, rostral prefrontal cortex; IC, insular cortex; oFC, orbitofrontal cortex; aCC, anterior cingulate cortex; aIC, anterior insular cortex; iCC, inter-calcarine cortex; mVC, medial visual cortex; MMSE, Mini-Mental State Examination.

interoceptive accuracy in general, was correlated with activity in the posterior right and left insula, right claustrum, precentral gyrus, and medial frontal gyrus. Thus, interoceptive accuracy, regardless of the type of task, is correlated with task-related activity in the insular cortex and ACC.

Recently, resting state fMRI studies have described the default mode network (DMN), executive control network (ECN), and SN as associated with high-level cognitive processes. Interoceptive-system hubs (e.g., ACC, insular cortex, and amygdala) overlap considerably with the SN (Kleckner et al., 2017). The results of the present study support the hypothesis that better performance on the heartbeat counting task is correlated with greater SN FC in older adults. The rostral prefrontal cortex seed was located in Brodmann's area 10, which is known to be involved in prospective memory tasks (Okuda et al., 1998), but in recent years, it has also been reported as related to pain (as pain matrix; Peng et al., 2018) and decision-making, in terms of complex information ethics (Sevinc et al., 2017). Sevinc et al. (2017) argued that the SN is implicated in the coordination of executive control and associative processes. Therefore, these results suggest that the rostral prefrontal cortex coordinates with the insular cortex, ACC, and orbitofrontal cortex to maintain interoceptive accuracy in older adults.

Differences Between Younger and Older Adults in the Neural Correlates of Interoceptive Accuracy

Chong et al. (2017) showed a positive correlation between the heartbeat counting score and SN FC of the right and left posterior insular cortices in healthy young adults, but no correlation was found for SN FC of the ACC. Moreover, no significant negative correlations were detected between the heartbeat counting score and SN FC. Critchley et al. (2001) proposed that changes in bodily states involve two hierarchical processes: a first-order context-independent autonomic representation within the insular and somatosensory cortices, and a second-order context- and experience-dependent representation within the cingulate and ventromedial prefrontal cortices. Thus, the results in Chong et al. (2017) indicate that interoceptive accuracy in young adults is more closely associated with context-independent autonomic representation than with context-dependent representation. In contrast to the study by Chong et al. (2017), the present study revealed a positive correlation between interoceptive accuracy and the ACC FC with the SN, using the same paradigm as that in Chong et al. (2017). Thus, the present study results suggest that context- and experience- dependent representation remains in older adults. Other studies have reported that, in older adults, context-dependent memory retrieval promotes the accuracy of a feeling of knowledge (Thomas et al., 2011), and that a protagonist's memorized personal experience promotes the accuracy of empathy (Wieck and Kunzmann, 2015).

Moreover, the Embodied Predictive Interoception Coding (EPIC) model (Kleckner et al., 2017) proposes that the interoceptive system has monosynaptic bidirectional connections between the insular cortex and ACC (as well as the dorsal amygdala) to exchange interoceptive predictions and prediction error signals. The EPIC model hypothesizes that the anterior insular cortex and ACC, as visceromotor regions, initiate visceromotor predictions through their cascading connections to

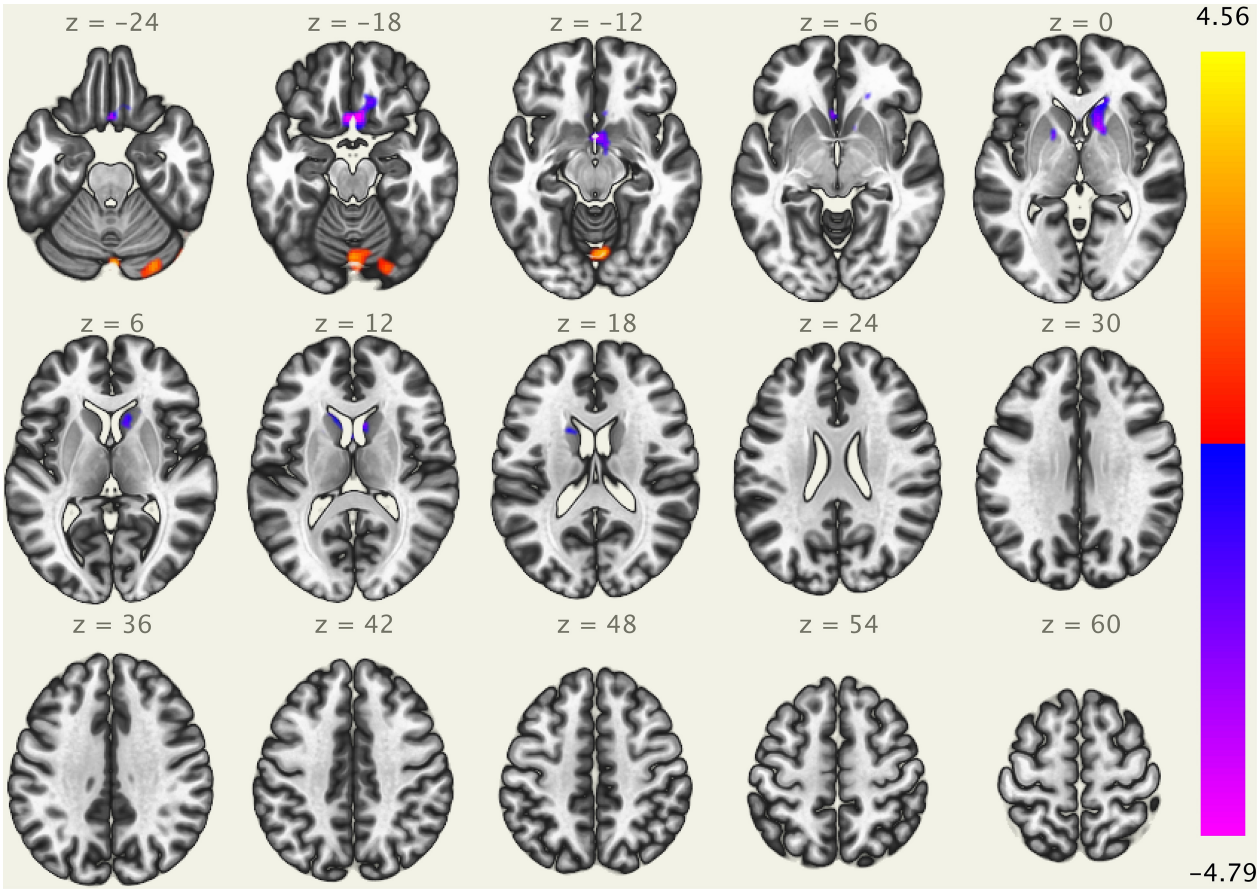

FIGURE 3 | Visual depiction of significant positive correlation (red) and negative correlation (blue) between voxel-based functional connectivity and interoceptive accuracy scores (after statistically accounting for age, sex, MMSE score, and GM volume). Interoceptive accuracy scores show significant positive correlation with the cerebellum (peak voxel = 56, –66, –36), and significant negative correlation with the lateral orbitofrontal cortex (peak voxel = 2, 18, –20) and caudate nucleus (peak voxel = 12, 14, –1); MMSE, Mini-Mental State Examination; GM, gray matter.

brain regions (e.g., hypothalamus) that control the body's internal milieu; simultaneously, the anterior insular cortex and ACC send information regarding the anticipated sensory consequences of interoceptive predictions to the primary interoceptive cortex (e.g., posterior insular cortex). Moreover, the primary interoceptive cortex receives ascending viscerosensory inputs; simultaneously, the posterior insular cortex sends information regarding the sensory consequences of interoceptive prediction errors to the anterior insular cortex and ACC. To summarize previous studies, context-dependent representation refers to the representation of predictions. Thus, the results of Chong et al. (2017) can be interpreted as showing a lack of representation of interoceptive predictions, which might, thus, explain why there was no correlation between interoceptive accuracy and ACC FC with the SN. Therefore, the existence of an association between interoceptive accuracy and ACC FC with the SN in the current study suggests that older adults maintain context-dependent representations, such as interoceptive predictions.

The results of this study suggest that the neural correlates of interoception accuracy differ between older and younger adults. The present study revealed that the left anterior insular cortex is correlated with regions associated with visual exteroception, such as the visual medial cortex and calcarine cortex, in older adults. Chong et al. (2017) reported no negative correlations between interoceptive accuracy and SN FC in younger adults. Previous studies reported that the anterior insular cortex may function as an important node for integrating information across multiple brain networks, such as the DMN and executive attention network (Uddin et al., 2014). In the present study, participants were asked to gaze at the fixation in the center of the screen via a mirror during the resting state scan. The negative connectivity between left anterior insular cortex and vision-related regions might represent negative feedback on vision-related exteroceptive processing to maintain interoceptive processing.

Previous studies reported that brain function or connectivity is decreased in posterior regions and increased in anterior regions in aging (Davis et al., 2008; Jockwitz et al., 2019; Ren et al., 2019). This phenomenon is defined as the Posterior–Anterior Shift in Aging (PASA) (Davis et al., 2008). The PASA model has been mainly used to explain the change in brain patterns involved in the direct linkage between the PASA phenomenon and the

cognitive aging process, as well as cognitive maintenance due to PASA. Previous studies reported that DMN FC is associated with cognitive function, and cognitive fatigue was related to the PASA phenomenon (Jockwitz et al., 2019; Ren et al., 2019). Moreover, Kehoe et al. (2013) examined age-related differences in functional reactivity to viewing pictures with high emotional arousal. In this previous study, older adults showed reduced reactivity in the bilateral occipital and temporal visual cortices, left inferior parietal cortex, and bilateral supplementary motor area compared to that in younger adults. The processing of emotional arousal information is largely associated with the SN; thus, the SN may be affected by the PASA phenomena. Future studies are needed to examine the existence of the PASA phenomenon in the SN.

Sex Differences in Interoceptive Accuracy

In the present study, there were no significant correlations among interoceptive accuracy, age, and cognitive function because the variances of age and MMSE score were small among the 27 participants. Moreover, the present study did not show a significant correlation between interoceptive accuracy and sex. Several earlier studies reported higher interoceptive accuracy scores in men than in women (Ludwick-Rosenthal and Neufeld, 1985; Montoya et al., 1993; Grabauskaitė et al., 2017). Grabauskaitė et al. (2017) suggested that one of the reasons males perform better than females on interoceptive accuracy tasks is the relevance of the biochemical properties of the heart (men have a larger heart capacity and a stronger heart muscle for systole than do women); thus, men are more likely to feel the heartbeat as a stimulus. While previous studies have focused on younger adults, the present study revealed no sex differences in interoceptive accuracy in older adults. These results indicate that sex differences in interoceptive accuracy may be affected by aging. However, higher interoceptive accuracy in women than in men has been reported in studies on mental disorders such as depression, somatic symptom disorder, and personality disorder (Mussgay et al., 1999) and in those with a small ratio of female participants. The present study had fewer male participants than female participants; since discrepancies in sex differences in interoceptive accuracy depend on the characteristics of the participants, careful consideration is required.

Time Counting Accuracy

In the present study, there was no significant relationship between heartbeat counting accuracy and time counting accuracy. Time counting accuracy reflects individual differences in timing accuracy, and is used as a control task to examine the validity of the heartbeat counting task (to check whether the counting of seconds is used when counting the heartbeats) (Schandry, 1981). The lack of a significant correlation in the present study suggests that participants did not refer to the number of seconds when counting heartbeats, indicating that the purpose of counting heartbeats was achieved. In addition, we excluded participants who could not perceive their own heartbeat without any cues and those with heartbeat counting accuracy $\pm$2SD or more from the mean, which may have contributed to the validity of the interoceptive counting accuracy.

Limitations

The present study has some limitations. First, the current study participants were female predominant (70%); thus, future studies are needed to examine the sex difference in interoceptive accuracy in a larger sample size with an equal gender ratio. Second, the current study did not examine age-related differences in the correlation between SN FC and individual differences in interoceptive accuracy. Future studies are needed to directly examine differences between older and younger adults. Third, this current study did not examine a questionnaire for interoception. Future studies are needed to examine the correlations or differences between interoceptive accuracy and awareness in functional brain connectivity.

Additionally, the present study used the heartbeat counting task. The heartbeat counting task is the most commonly utilized method for assessing the perception of heartbeat sensations; however, approximately 40% of participants are reportedly not able to consciously register their heartbeats at all (Khalsa et al., 2009a). Khalsa et al. (2009a) proposed a novel protocol based on the standardized isoproterenol sensitivity test, which involves multiple bolus administrations of isoproterenol. Furthermore, another study (Fittipaldi et al., 2020) examined a convergent multidimensional and multi-feature approach to interoception using a novel interoceptive accuracy index (IA-md) based on heartbeat detection tasks. The authors have reported that the IA-md is associated with the electroencephalogram-derived heart-evoked potential, FC of interoceptive seeds (including bilateral insular cortex, ACC, and postcentral cortex), and performance in an emotional face recognition task. Future studies are warranted to further evaluate measuring methods of interoceptive accuracy to develop a comprehensive view of the functional integrity of interoception.

CONCLUSION

The current results provide new insights into the relationship between intrinsic SN FC and individual differences in interoceptive accuracy in older adults. The present study showed that interoceptive accuracy is positively correlated with connectivity within the SN and negatively correlated with connectivity between the left insular cortex and occipital cortex during rest in older adults. These findings suggest that the positive correlation between left rostral prefrontal cortex and the SN and the negative correlation between left insular cortex and vision-related exteroceptive brain regions aid in maintaining interoceptive accuracy. Moreover, in reference to previous results in younger adults (Chong et al., 2017), the observed positive correlation among SN might be a comprehensive interoceptive process by several brain regions in older adults. Moreover, the observed negative correlation between left insular cortex and occipital cortex in the interoceptive process might control exteroceptive information in older adults. These results suggest

that the PASA phenomenon occurs in the SN to maintain interoceptive accuracy in older adults.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kyoto Prefectural University of Medicine (ERB-C-853-3). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DU: execution of the research project, data collection, statistical analysis, writing the first draft, and manuscript review and critique. TM: execution of the research project, data collection, statistical review and critique, and manuscript review and critique. YK: execution of the research project and manuscript review and critique. NA: execution of the research project, statistical review and critique, and manuscript review and critique. SM and MT: data collection and manuscript review and critique. JN: organization and execution of the research project, statistical review and critique, and manuscript review and critique. All authors read and approved the final manuscript.

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Regional Gray Matter Volume Identifies High Risk of Unsafe Driving in Healthy Older People

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In developed countries, the number of traffic accidents caused by older drivers is increasing. Approximately half of the older drivers who cause fatal accidents are cognitively normal. Thus, it is important to identify older drivers who are cognitively normal but at high risk of causing fatal traffic accidents. However, no standardized method for assessing the driving ability of older drivers has been established. We aimed to establish an objective assessment of driving ability and to clarify the neural basis of unsafe driving in healthy older people. We enrolled 32 healthy older individuals aged over 65 years and classified unsafe drivers using an on-road driving test. We then utilized a machine learning approach to distinguish unsafe drivers from safe drivers based on clinical features and gray matter volume data. Twenty-one participants were classified as safe drivers and 11 participants as unsafe drivers. A linear support vector machine classifier successfully distinguished unsafe drivers from safe drivers with 87.5% accuracy (sensitivity of 63.6% and specificity of 100%). Five parameters (age and gray matter volume in four cortical regions, including the left superior part of the precentral sulcus, the left sulcus intermedius primus [of Jensen], the right orbital part of the inferior frontal gyrus, and the right superior frontal sulcus), were consistently selected as features for the final classification model. Our findings indicate that the cortical regions implicated in voluntary orienting of attention, decision making, and working memory may constitute the essential neural basis of driving behavior.

Keywords: gray matter volume, healthy older people, machine learning, on-road driving, support vector machine, unsafe driving

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INTRODUCTION

Driving requires the integration of sensory, motor, and cognitive functions (Hird et al., 2016). Because these functions typically decline with age, older people are at an increased risk of causing fatal traffic accidents (Anstey et al., 2005). In developed countries, the number of traffic accidents caused by older drivers is increasing over time, along with the aging population. In Japan, the number of licensed drivers aged over 65 years has increased in recent years, exceeding 18 million in 2018, and the number of fatal accidents caused by older drivers has maintained an upward trend (Ichikawa et al., 2020). Although previous studies consistently showed that individuals

with dementia are at increased risk of unsafe driving among the older population (Drachman and Swearer, 1993; Frittelli et al., 2009), surprisingly, a report published by the National Police Agency revealed that approximately half of the older drivers who caused fatal accidents were cognitively normal (National Police Agency, 2020). These findings suggest that detecting dementia and mild cognitive impairment (MCI) is not sufficient to prevent fatal traffic accidents caused by older drivers.

Despite the importance of identifying characteristics of older drivers who are cognitively normal but at high risk of causing fatal traffic accidents, a standardized method for assessing the driving ability of healthy older drivers has not yet been established. Although on-road driving tests are recognized as the gold standard assessment for measuring driving ability, it is not practical to perform driving tests for all older drivers because of the cost involved (Langford et al., 2004). One previous structural magnetic resonance imaging (MRI) study including both healthy older people and those with MCI reported that gray matter volume in premotor cortex was negatively correlated with the tendency to commit driving errors assessed with the Driving Behavior Questionnaire (DBQ) (Sakai et al., 2012). This finding suggests that biological markers of unsafe driving might be captured by brain MRI; however, no previous study has assessed driving ability and investigated the neural correlates of driving ability among healthy older people.

The current study had two main aims. First, we categorized participants into unsafe or safe drivers using a new sensing method for the objective evaluation of on-road driving ability of healthy older people on the basis of vehicle behavior using a data recorder and video cameras. Second, to describe the neurobiological features associated with unsafe driving, we built a classification model to distinguish unsafe from safe drivers based on gray matter volume data using a linear support vector machine (SVM) approach.

MATERIALS AND METHODS

Participants

The present study recruited 32 healthy older individuals aged over 65 years from the local community through online advertisements at the University of Tokyo and the Musashisakai Driving School (Tokyo, Japan). All participants were diagnosed as "cognitively normal" using the Alzheimer's Disease Neuroimaging Initiative (ADNI) diagnostic classification: (1) Mini-Mental State Examination (MMSE) score between 24 and 30; (2), Clinical Dementia Rating (CDR) score of 0; (3) normal memory function measured by education-adjusted scores on the Logical Memory II subscale of delayed paragraph recall from the Wechsler Memory Scale—Revised (WMS-R) (Petersen et al., 2010). Participants were confirmed to have had no lifetime history of diagnoses of psychiatric or neurological conditions. Participants were also required to maintain a current valid driver's license and to still be actively driving at the time of the study. After an extensive description of the study, written informed consent was obtained from all participants prior to enrollment and investigations were performed in accordance with the ethical standards of the Declaration of Helsinki. The study protocol was approved by the ethics committees of the University of Tokyo and Keio University. After all participants took an on-road driving test at the Musashisakai Driving School, they were moved to Keio University Hospital and underwent cognitive assessments, a visual function test, and an MRI scan.

Measurements

Cognitive Assessment

The general cognitive function of each participant was assessed using the Raven's Colored Progressive Matrices (RCPM). The Rey Auditory Verbal Learning Test (RAVLT), the Rey–Osterrieth Complex Figure Test (ROCFT), the Clock Drawing Test (CDT), and the Everyday Memory Checklist (EMC) were used to evaluate memory and visuospatial function. We estimated attentional/executive function using the Stroop Test (ST), the Trail Making Test (TMT) A and B, and the Dysexecutive Questionnaire (DEX). We investigated subjective driving ability using the DBQ. Depression severity was evaluated using the Geriatric Depression Scale (GDS). Handedness was assessed using the Edinburgh Handedness Inventory (Oldfield, 1971). The CDT was scored using a five-point scoring system adopted from the ADNI's cognitive assessments (Iwatsubo et al., 2018). Clinical neuropsychologists (MY and KK) administered all cognitive assessments in an environment with adequate lighting and reduced noise conditions.

Cambridge Neuropsychological Test Automated Battery

In addition to these neuropsychological tests mentioned above, we performed the Cambridge Neuropsychological Test Automated Battery (CANTAB), which is a computer-based test battery widely used in neurocognitive studies (Cambridge Cognition, Cambridge, United Kingdom) (Robbins et al., 1994). Specifically, we adopted the CANTAB battery consisting of four cognitive domain tasks to assess subtle cognitive changes with aging (Soares et al., 2015): (1) visual memory (paired associates learning [PAL]); (2) attention (reaction time [RTI]); (3) working memory (spatial working memory [SWM]); (4) control task measuring simple psychomotor speed and accuracy (motor screening task [MOT]). According to the standard protocol, the instructions for the tests were explained to the participants before initiation of the study. The standard instructions for the tests were provided in the CANTAB manual and were translated into Japanese. The execution of the tasks required approximately 20 min. The test battery was administered in a silent room without distractions. Details of the procedures are available elsewhere (Akter et al., 2015).

Functional Visual Acuity Test

We adopted binocular functional visual acuity to assess the visual function associated with driving ability. First, we measured corrected distance visual acuity (CDVA) using Landolt vision charts. Second, we calculated corrected distance functional visual acuity (CDFVA) with the AS-28 FVA Measurement System (Kowa, Aichi, Japan) (Katada et al., 2016; Negishi et al., 2016). CDFVA consists of five indicators: functional visual acuity

(FVA), maximum visual acuity (MaxVA), minimal visual acuity (MinVA), visual maintenance ratio (VMR), and average response time (ART). FVA, MaxVA, and MinVA represent the average, maximum, and minimum visual acuity in a period of 60 s, respectively. VMR was defined as the ratio of FVA to CDVA. ART was computed as the average response time in giving the direction of a Landolt ring, which was shown on the screen every 2 s.

Image Acquisition and Preprocessing

MR images were acquired using a 3.0-T MRI scanner (MAGNETOM Verio, Siemens Healthineers, Erlangen, Germany) with an 8-channel head coil. High-resolution T1-weighted images were acquired using a magnetization-prepared rapid acquisition with gradient echo sequence (repetition time: 1.9 s; echo time: 2.99 ms; flip angle: 9°; field of view: 256 mm; matrix size: 256 × 256; slice thickness: 1.2 mm; 192 sagittal slices; voxel size: 1 × 1 × 1.2 mm). All images were first visually checked for scanner artifacts and anatomical anomalies.

Structural MRI data were preprocessed using FreeSurfer's *recon-all* processing pipeline for cortical reconstruction and volumetric segmentation (Fischl and Dale, 2000; Fischl et al., 2004) (software freely available at http://surfer.nmr.mgh.harvard.edu/). The cortical processing stream in FreeSurfer included Talairach transformation, removal of non-brain tissue, segmentation of subcortical white matter and gray matter tissue, intensity normalization and atlas registration. After these automatic steps, a triangular mesh model of the cortical surface consisting of over 150,000 vertices per hemisphere was generated, and the cortical surface was parcellated into 74 distinct cortical regions of interest (ROIs) based on curvature values of the surface for each hemisphere according to the Destrieux atlas (Destrieux et al., 2010). Each preprocessed image was visually inspected and any segmentation errors were manually corrected by a researcher. Gray matter volume for each ROI was then calculated automatically using FreeSurfer's recon-all processing pipeline. Furthermore, ROI gray matter volumes were divided by each subject's estimated total intracranial volume (eTIV) to adjust for individual differences in overall cranial size (O'Brien et al., 2011).

On-Road Driving Test

To evaluate the on-road driving ability of healthy older individuals on the basis of vehicle behaviors, we used an instrumented automatic vehicle with a data recorder, charge-coupled device (CCD) cameras, and dual brake controls (**Figures 1A,B**; Shino et al., 2018). The data recorder (Tough More-eye S manufactured by Finefit Design, Aichi, Japan) provides vehicle information, including speed, acceleration, and gas and brake pedal positions. CCD cameras filmed the driver's face, gaze, and footwork and surrounding traffic and lanes. The position and location of the vehicle was determined using data from these cameras. On-road driving tests were performed at the Musashisakai Driving School in suburban Tokyo. All participants drove the instrumented vehicle on the same course in a city area around the driving school for 30 min. To ensure participant safety, a driving instructor accompanied participants in the car while they drove the vehicle.

Classification of Safe and Unsafe Drivers

We evaluated participants' driving ability at intersections with a stop sign using on-road driving test data, because older drivers most frequently cause traffic accidents at intersections without traffic lights (Cicchino and McCartt, 2015; Lombardi et al., 2017). At intersections with a stop sign, drivers generally need to notice the stop sign, slow down the vehicle sufficiently, then pass through the intersection without inappropriate acceleration. More precisely, drivers must decelerate the vehicle from the stop line to the entrance of the intersection sufficiently to be able to stop immediately if there are other cars or pedestrians at the intersection, and should not accelerate at the entrance of the intersection. We thus evaluated these driving behaviors at intersections using two parameters: the minimum speed of a vehicle moving past the stop line to the entrance of the intersection, and the speed of the vehicle at the entrance of the intersection (see details in **Figure 1C**). Specifically, we classified all participants into unsafe and safe drivers according to two criteria: (1) whether the minimum speed of the vehicle between the stop line and the entrance of the intersection was less than 5 km/h; (2) whether the speed of the vehicle was less than 5 km/h when the front of the car was at the entrance of the intersection. We classified participants who met both criteria as safe drivers, while we classified those who did not as unsafe drivers (see details in **Figure 1D**). We used a cut-off value of 5 km/h to divide participants into unsafe and safe drivers because automatic vehicles are designed to move forward at a speed of less than 5 km/h when drivers release the brake pedal without depressing the gas pedal (Nesamani and Subramanian, 2006; Seers et al., 2015). A previous study analyzing more than 8,000 traffic accidents reported that no serious accidents occurred when the speed of the vehicle was below 5 km/h (Kröyer, 2015). Further, we adopted the same cut-off value (5 km/h) in our previous study assessing the driving ability of older people based on the speed of the vehicle at intersections (Shino et al., 2018; Yamamoto et al., 2020). Therefore, in the present study, participants who drove a vehicle below 5 km/h at the intersection were classified as safe drivers.

Classification Using Machine Learning

The purpose of the current study was to build a classification model to dissociate unsafe drivers from safe drivers using a machine learning. Based on a previous study suggesting that combining neuroimaging and clinical data could improve the accuracy of predicting cognitive decline (Lahmiri and Shmuel, 2019), we made a classification model using both gray matter volume data and clinical measures. A total of 56 clinical features and a total of 148 parcellated cortical regions were included for the classification model. Further, before we created the classification model, we attempted to overcome the issue of a small sample size for classification by selecting features used in the classification model with the least absolute shrinkage and selection operator (LASSO) algorithm. In general, the LASSO algorithm performed linear regression with L1-regularization and conducted feature selection based on the regularization parameter α. In the current study, the optimal value of the regularization parameter α was achieved using the Akaike

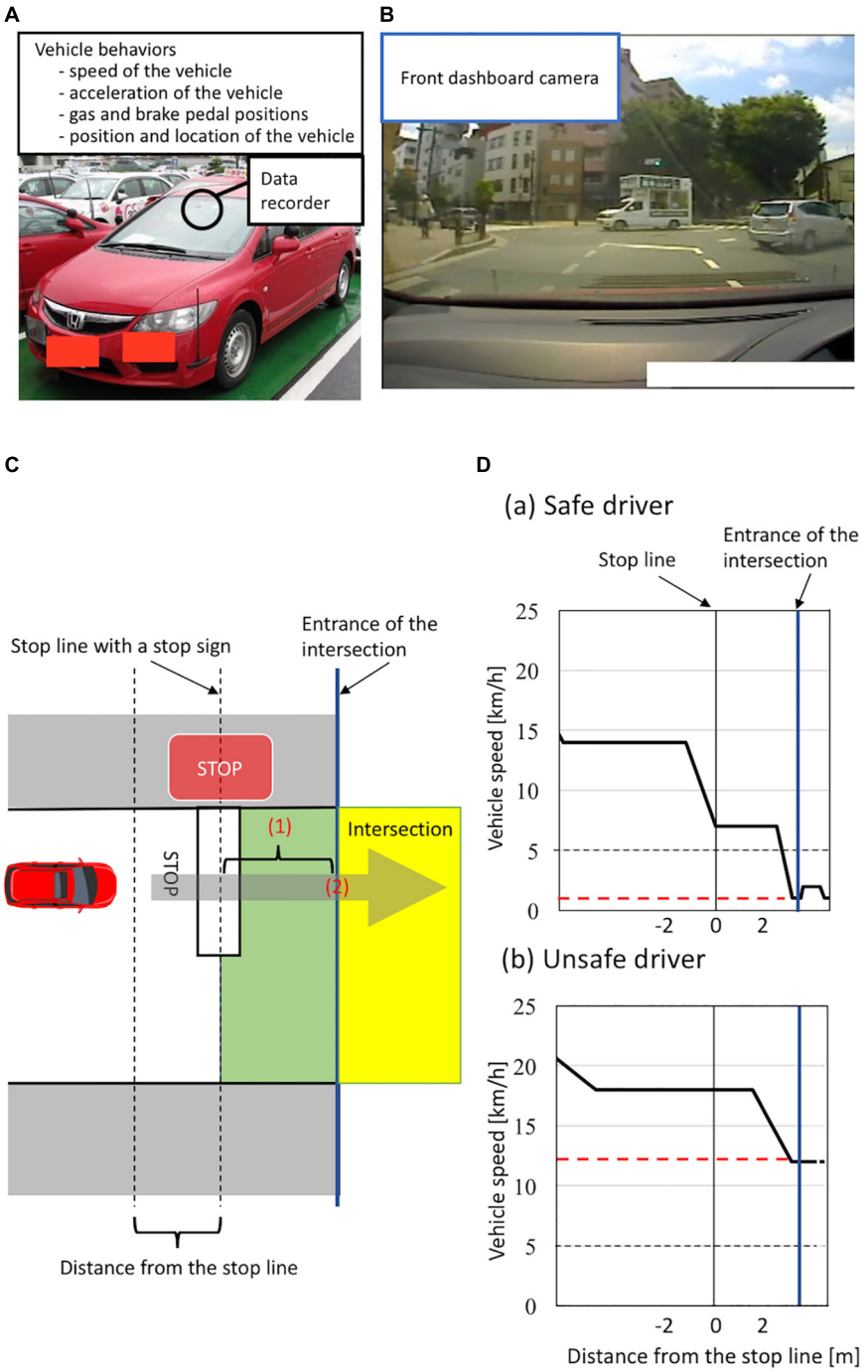

FIGURE 1 | (A–B) Instrumented automatic vehicle for evaluating vehicle behaviors using a data recorder (Tough More-eye S manufactured by Finefit Design, Aichi, Japan) and charge-coupled device (CCD) cameras. **(A)** The data recorder provides the speed and acceleration of the vehicle and the positions of gas and brake pedal. The data recorder was placed on the front dashboard. **(B)** The front dashboard CCD camera filmed surrounding traffic and lanes. **(C–D)** Classification of a safe or unsafe driver based on vehicle behaviors. **(C)** The definition of the area of the intersection having a stop sign. Vehicles are driven on the left side of the road in Japan. The area from the stop line to the entrance of the intersection is shown in green. The area of the intersection is shown in yellow. The entrance of the intersection is shown by a blue line. To evaluate drivers' behavior, we measured (1) the minimum speed of the vehicle between the stop line and the entrance of the intersection and (2) the speed of the vehicle when the front of the car was at the entrance of the intersection. **(D)** Schema of velocity distribution patterns measured using the instrumented vehicle. Figures show examples of velocity distribution patterns of (a) a safe driver and (b) an unsafe driver. The minimum speed of a vehicle from the stop line to the entrance of the intersection is shown by a red line. We classified all participants into two groups according to (1) whether the minimum speed of the vehicle between the stop line and the entrance of the intersection was less than 5 km/h and (2) whether the speed of the vehicle when the front of the car was at the entrance of the intersection was less than 5 km/h. We classified participants who met both criteria as safe drivers, and classified those who did not as unsafe drivers.

Information Criterion, which is widely used as a penalized likelihood criterion (Congdon, 2010).

In the present study, a linear SVM was used to create the classification model. The linear SVM method is widely used as a powerful supervised learning methodology in binary classification (Cortes and Vapnik, 1995). In general, linear SVM classifiers separate two groups based on the value of penalty coefficient C, which determines the learning algorithm for classification. In the current study, the optimal value of the penalty coefficient C was tuned using Optuna, which is a hyperparameter optimization framework applicable to machine learning (Akiba et al., 2019).

After fixing C for the linear SVM classifier, we evaluated the performance of the linear SVM classifier using leave-one-out cross-validation (LOOCV), which is a widely used validation method for accurately assessing the performance of predictive models. Specifically, LOOCV maximizes the training sample and avoids possible case partition bias, even with small sample sizes (Lopes et al., 2019). In our study, LOOCV continued for 32 rounds to test all samples one by one. At each round of LOOCV, one participant was selected as testing data, and the remaining 31 participants were used to train the linear SVM classifier. After 32 rounds, the accuracy, sensitivity, and specificity of the linear SVM classifier were estimated.

We used scikit-learn in Python 3.7.0 for machine-learning analyses (Pedregosa et al., 2011).

Statistical Analysis

For clinical data, we adopted a two-tailed *t*-test, chi-squared test, or multivariate analysis of variance in the group comparison between safe drivers and unsafe drivers. IBM SPSS software Statistics 25 for Mac OS (IBM, Armonk, NY) was used for the statistical analysis. We used a liberal statistical threshold of $P < 0.05$.

RESULTS

Demographic Characteristics, Neuropsychological, and Functional Visual Acuity Tests

We classified 21 participants as safe drivers and 11 participants as unsafe drivers (**Table 1**). There were significant differences between groups in the DEX, EMC, PAL total errors (six shapes, adjusted), and RTI simple accuracy scores. There were no differences in the sex ratio, age, duration of education, driving experience, handedness, and results of functional visual acuity test between the groups.

Linear SVM Classifier for Unsafe Driving Using Gray Matter Volume and Clinical Features

The linear SVM classifier ($\alpha = 0.043$, $C = 0.027$) using clinical features and gray matter volume data distinguished unsafe drivers from safe drivers with an accuracy of 87.5% (sensitivity of 63.6% and specificity of 100%). While 36 parameters were selected as features for the final classification model at least once throughout cross-validation procedures (**Figure 2**), five parameters (age and gray matter volume of four cortical regions, including the left superior part of the precentral sulcus, the left sulcus intermedius primus [of Jensen], the right orbital part of the inferior frontal gyrus, and the right superior frontal sulcus) were consistently selected at every iteration (**Figure 3**). In an additional analysis, when we selected only these five parameters as input data for classification, the linear SVM classifier ($C = 0.050$) successfully differentiated unsafe drivers from safe drivers with accuracy of 87.5% (sensitivity of 81.8% and specificity of 90.5%).

DISCUSSION

In the present study, the linear SVM classifier using both clinical features and gray matter volume data differentiated unsafe drivers from safe drivers with an accuracy of 87.5% (sensitivity of 63.6%, and specificity of 100%). Furthermore, in the final classification model, age and gray matter volume in four cortical regions, including the left superior part of the precentral sulcus, the left sulcus intermedius primus (of Jensen), the right orbital part of the inferior frontal gyrus, and the right superior frontal sulcus, were selected as consistent features, suggesting that regional gray matter volume changes in these four cortical regions are strongly associated with a high risk of unsafe driving among healthy older people.

One advantage of the present study is that we objectively evaluated on-road driving behaviors. In previous structural MRI studies investigating the neural basis of driving ability among older people, interviews or questionnaires were often utilized for driving evaluation (Sakai et al., 2012; Park et al., 2013; Jang et al., 2018). Furthermore, even in standardized on-road driving tests, such as Iowa's driving test, scores are provided by a driving instructor (Dawson et al., 2009). We therefore consider that these measurements may not accurately estimate a participant's driving ability because the actual vehicle's behaviors on the road were not assessed. To overcome these methodological issues, we assessed driving ability according to vehicle behaviors during on-road driving test using an instrumented automatic vehicle with a data recorder and CCD cameras (Shino et al., 2018; Yamamoto et al., 2020). Specifically, focusing on driving behaviors at intersections, we evaluated participants' driving ability using the actual speed of the vehicle at intersections, because older drivers most frequently cause traffic accidents at intersections (Cicchino and McCartt, 2015; Lombardi et al., 2017).

Our final classification model successfully dissociated unsafe drivers from safe drivers with 87.5% accuracy. We consider that the accuracy of our model was relatively high, because in two previous studies using only neuropsychological tests to predict driving ability, prediction accuracies were 66 and 90%, respectively (Brown et al., 2005; Ott et al., 2008). Furthermore, our final model identified age and gray matter volume of four cortical regions as consistent features for classification. In an additional analysis using only these five features, our model successfully dissociated unsafe drivers from safe drivers with

87.5% accuracy. This finding further suggested that the driving ability of healthy older people could be predicted accurately using only information about age and MRI data. Given that both age and MRI data are rater-independent variables, our classification model appears to be reliable, with a range of potential clinical applications.

TABLE 1 | Demographics and results of neuropsychological and functional visual acuity test.

Demographic data					
		Safe drivers	**Unsafe drivers**	***F* or *T***	***P*-value**
n		21	11		
Sex male/female		20/1	10/1		0.631
Age (years)		74.9 ± 3.7	77.9 ± 4.1	2.02	0.052
Handedness		89.4 ± 34.3	100 ± 0.0	0.99	0.330
Education (years)		14.4 ± 2.1	14.5 ± 1.9	0.10	0.925
Driving experience (years)		51.0 ± 6.9	47.0 ± 14.5	0.83	0.424
Neuropsychological tests					
	Subscale	**Safe drivers**	**Unsafe drivers**	***F* or *T***	***P*-value**
MMSE total		27.5 ± 2.2	27.8 ± 1.5	0.38	0.708
Logical memory of the WMS-R				0.54	0.586
	Immediate recall	19.4 ± 5.1	17.2 ± 7.4		
	Delayed recall	15.1 ± 5.4	12.8 ± 6.2		
RCPM		29.3 ± 2.9	31.0 ± 2.9	1.53	0.135
RAVLT				1.58	0.183
	Immediate recall, 1st trial	5.2 ± 1.8	4.2 ± 1.5		
	Immediate recall, 2nd trial	7.2 ± 1.9	7.2 ± 2.0		
	Immediate recall, 3rd trial	8.8 ± 2.4	8.5 ± 1.8		
	Immediate recall, 4th trial	9.8 ± 2.6	10.0 ± 2.0		
	Immediate recall, 5th trial	10.8 ± 2.3	10.5 ± 2.3		
	Interference	4.6 ± 1.5	4.3 ± 1.8		
	Delayed recall	8.8 ± 3.1	6.7 ± 3.5		
	Recognition correct	14.0 ± 0.9	13.2 ± 3.9		
	Recognition false positive	1.1 ± 1.8	0.6 ± 1.1		
	Recognition false negative	1.0 ± 0.9	1.8 ± 3.9		
ROCFT				1.98	0.156
	Copy	35.0 ± 1.3	35.5 ± 0.8		
	Delayed recall	20.0 ± 5.0	23.9 ± 5.0		
ST	Completion time			0.64	0.598
	Part I (s)	17.1 ± 2.7	18.1 ± 3.8		
	Part II (s)	20.0 ± 3.8	21.8 ± 4.6		
	Part III (s)	28.8 ± 11.2	29.3 ± 6.3		
	Numbers of errors			0.32	0.813
	Part I	0.1 ± 0.3	0.1 ± 0.3		
	Part II	0.2 ± 0.5	0.3 ± 0.4		
	Part III	1.2 ± 1.4	0.7 ± 1.1		
TMT				0.55	0.585
	A	100.7 ± 33.9	97.3 ± 20.2		
	B	158.9 ± 79.2	133.8 ± 55.9		
CDT				0.77	0.388
	Copy	5.0 ± 0.0	5.0 ± 0.0		
	Free-drawn	4.9 ± 0.3	4.7 ± 0.4		
DEX*		10.5 ± 7.3	17.2 ± 9.4	2.15	0.040
EMC*		6.7 ± 3.8	10.8 ± 3.8	2.82	0.008
DBQ		69.5 ± 14.5	73.2 ± 15.2	0.65	0.518
GDS		1.3 ± 1.5	2.5 ± 2.3	1.68	0.104

(Continued)

TABLE 1 | Continued

Cambridge Neuropsychological Test Automated Battery					
	Subscale	**Safe drivers**	**Unsafe drivers**	***F* or *T***	***P*-value**
MOT					
	Latency			1.64	0.211
	Mean	799.0 ± 122.3	902.4 ± 275.1		
	Median	780.1 ± 132.8	807.2 ± 143.0		
	Mean error	10.6 ± 2.8	8.7 ± 2.3	1.85	0.075
PAL					
	TE (adjusted)	36.5 ± 23.9	32.4 ± 18.9	0.49	0.631
	TE (six shapes, adjusted)*	9.5 ± 7.6	3.6 ± 3.3	2.35	0.025
RTI					
	Simple				
	Accuracy score*	8.7 ± 0.5	9.0 ± 0.0	2.34	0.030
	Reaction time			1.41	0.261
	Mean	307.4 ± 46.5	284.1 ± 23.6		
	Median	293.4 ± 40.5	278.2 ± 25.6		
	SD	53.5 ± 32.9	31.5 ± 9.6		
	Movement time			1.06	0.382
	Mean	411.2 ± 119.9	408.1 ± 66.7		
	Median	403.6 ± 118.1	401.5 ± 63.8		
	SD	51.4 ± 25.9	37.5 ± 12.3		
	5 Choice				
	Accuracy score	7.9 ± 0.3	7.9 ± 0.3	0.04	0.969
	Reaction time			0.20	0.896
	Mean	342.1 ± 33.7	347.9 ± 45.5		
	Median	337.4 ± 36.2	345.5 ± 39.3		
	SD	43.3 ± 19.5	43.5 ± 20.0		
	Movement time			0.37	0.773
	Mean	431.9 ± 105.7	404.9 ± 73.2		
	Median	432.4 ± 106.4	401.7 ± 79.0		
	SD	39.0 ± 14.6	40.3 ± 30.1		
SWM					
	Between errors	48.3 ± 16.9	43.7 ± 11.0	0.79	0.434
	Strategy	36.8 ± 4.0	36.0 ± 2.1	0.61	0.546

Functional visual acuity test				
	Safe drivers	**Unsafe drivers**	***F* or *T***	***P*-value**
FVA (logMAR)	0.123 ± 0.132	0.216 ± 0.148	1.77	0.088
MaxVA (logMAR)	−0.019 ± 0.123	0.066 ± 0.104	1.90	0.067
MinVA (logMAR)	0.300 ± 0.217	0.385 ± 0.227	1.01	0.318
VMR	0.93 ± 0.06	0.92 ± 0.08	0.45	0.662
ART	1.44 ± 0.11	1.41 ± 0.08	0.67	0.511

MMSE, Mini-Mental State Examination; WMS-R, Logical Memory II subscale of the Wechsler Memory Scale—Revised; RCPM, Raven's Colored Progressive Matrices; RAVLT, Rey Auditory Verbal Learning Test; ROCFT, Rey–Osterrieth Complex Figure Test; ST, Stroop Test; TMT, Trail Making Test; CDT, Clock Drawing Test; DEX, Dysexecutive Questionnaire; EMC, Everyday Memory Checklist; DBQ, Driving Behavior Questionnaire; GDS, Geriatric Depression Scale; MOT, motor screening task; PAL TE, paired associates learning total error; RTI, reaction time; SWM, spatial working memory; FVA, Functional visual acuity; MaxVA, Maximal functional visual acuity; MinVA, Minimal functional visual acuity; VMR, Visual maintenance ratio; ART, Average response time; logMAR, Logarithm of the minimum angular resolution; SD, standard deviation. Asterisks indicate statistical significance between groups ($P < 0.05$).

The current results revealed that the risk of unsafe driving increases with age. A recent meta-analysis of global longitudinal cohort data revealed that all cognitive domains, particularly attentional function, decline with age (Lipnicki et al., 2017). Driving ability is also reported to be affected by aging. For example, a 2-year longitudinal study that observed the change in driving ability in older people described a gradual decline in driving ability (Duchek et al., 2003). Similarly, a recent large-sample study among older people concluded that age was the most consistent predictor of on-road driving ability (Anstey et al., 2017). However, because all of these previous studies included both healthy people and people with MCI in their analyses, the

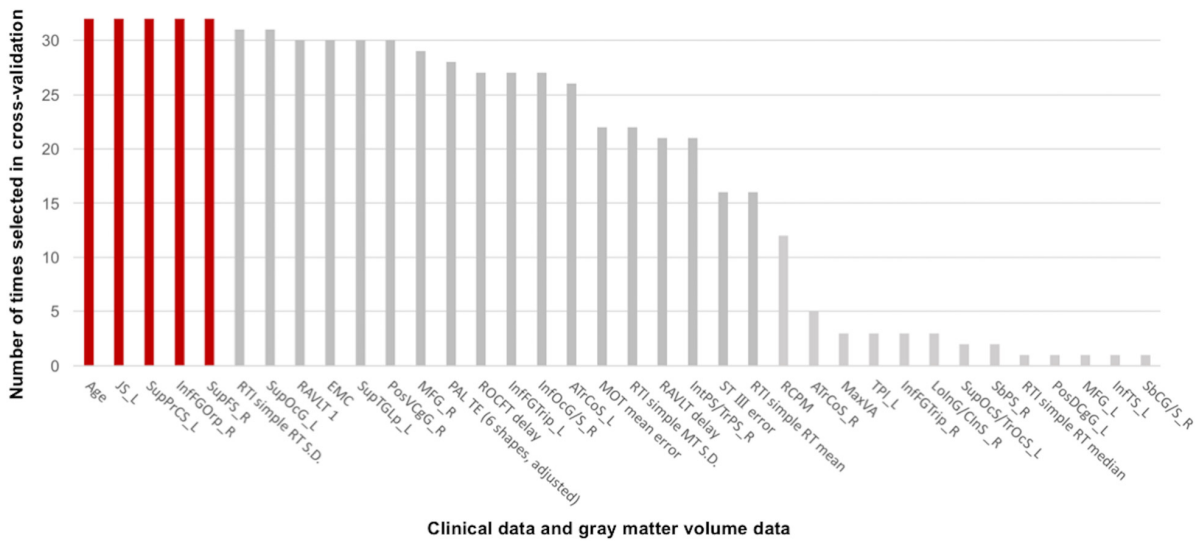

FIGURE 2 | Contributions of clinical data and gray matter volume data to the classification of safe and unsafe drivers in the final model. The number of times each parameter was selected in the cross-validation is shown for all 36 parameters. Higher numbers represent a greater contribution to the classifier. Five parameters (age and gray matter volume of four cortical regions, including the left superior part of the precentral sulcus, the left sulcus intermedius primus [of Jensen], the right orbital part of the inferior frontal gyrus, and the right superior frontal sulcus) were consistently selected at every iteration. JS_L, the left sulcus intermedius primus (of Jensen); SupPrCS_L, the left superior part of the precentral sulcus; InfFGOrp_R, the right orbital part of the inferior frontal gyrus; SupFS_R, the right superior frontal sulcus; RTI, reaction time; SupOcG_L, the left superior occipital gyrus; RAVLT 1, the first trial of the Rey Auditory Verbal Learning Test immediate recall; EMC, Everyday Memory Checklist; SupTGLp_L, the left lateral aspect of the superior temporal gyrus; PosVCgG_R, the right posterior-ventral part of the cingulate gyrus; MFG_R, the right middle frontal gyrus; PAL TE, paired associates learning total error; ROCFT delay, Delayed recall of the Rey–Osterrieth Complex Figure Test; InfFGTrip_L, the left triangular part of the inferior frontal gyrus; InfOcG/S_R, the right inferior occipital gyrus and sulcus; ATrCoS_L, the left anterior transverse collateral sulcus; MOT, motor screening task; RAVLT delay, Delayed recall in the Rey Auditory Verbal Learning Test; IntPS/TrPS_R, the right intraparietal sulcus (interparietal sulcus) and transverse parietal sulci; ST III, Time taken to finish the Stroop Test part III; RCPM, Raven's Colored Progressive Matrices; ATrCoS_R, the right anterior transverse collateral sulcus; MaxVA, Maximal functional visual acuity; TPl_L, the left temporal plane of the superior temporal gyrus; InfFGTrip_R, the right triangular part of the inferior frontal gyrus; LoInG/ClnS_R, the right long insular gyrus and central insular sulcus; SupOcS/TrOcS_L, the left superior occipital sulcus and transverse occipital sulcus; SbPS_R, the right subparietal sulcus; PosDCgG_L, the left posterior-dorsal part of the cingulate gyrus; MFG_L, the left middle frontal gyrus; InfTS_L, the left inferior temporal sulcus; SbCG/S_R, the right subcentral gyrus (central operculum) and sulci.

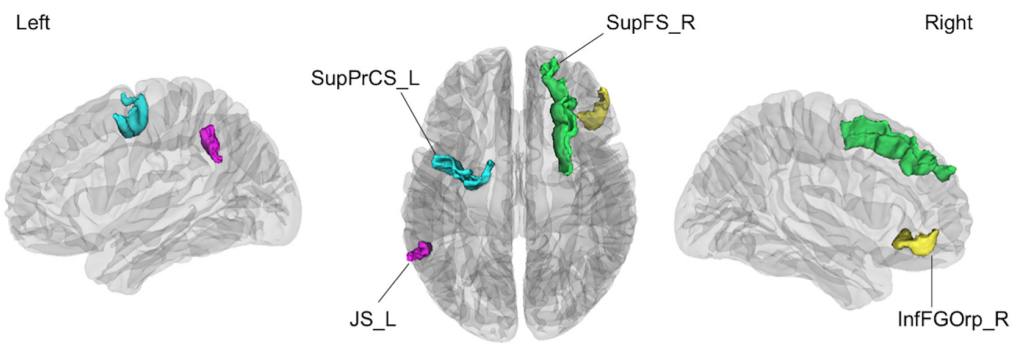

FIGURE 3 | The four cortical regions identified as consistent classification inputs were located within the cortical regions involved in cognitive functions essential for driving, such as voluntary orienting of attention, decision making, and working memory. SupPrCS_L, the left superior part of the precentral sulcus; JS_L, the left sulcus intermedius primus (of Jensen); InfFGOrp_R, the right orbital part of the inferior frontal gyrus; SupFS_R, the right superior frontal sulcus.

effect of age on driving ability in healthy older people was not evident. The present results therefore expand on prior findings to the extent that even in cognitively normal older people, there is a strong relationship between unsafe driving and aging.

The current data revealed that regional gray matter volume changes are highly predictive of driving ability in healthy older people. This finding suggests that gray matter volume accurately reflects changes in cortical structure related to decreased driving ability among healthy older people. One previous study examining the association of cortical changes with driving ability among older people reported that gray matter volume was correlated with driving ability, supporting the current results (Sakai et al., 2012). In the present study, four cortical regions (the left superior part of the precentral sulcus, the left sulcus

intermedius primus [of Jensen], the right orbital part of the inferior frontal gyrus, and the right superior frontal sulcus) were identified as consistent classification inputs to dissociate unsafe drivers from safe drivers using the linear SVM.

The superior part of the precentral sulcus is located in the dorsal premotor cortex (PMC) including the frontal eye field (FEF). The FEF plays a decisive role in saccade programming and shows enhanced responses to a visual stimulus when it is the saccade target (Ptak and Schnider, 2010). The sulcus intermedius primus (of Jensen) is located in the inferior parietal lobule (IPL), including the supramarginal and angular gyri, which is involved in visual attention or motion perception (Zhang and Li, 2014). Importantly, the dorsal attention network (DAN) linking the FEF with the IPL is involved in voluntary orienting of visuospatial attention (Ptak, 2012; Tamber-Rosenau et al., 2018). Furthermore, the DAN improves target detection and behavioral performance by activating the visual cortex prior to the appearance of the target, particularly during anticipatory attention, in which advanced information is utilized to orient visuospatial attention to the location of an impending target, such as a road sign (Bressler et al., 2008). The DAN thus plays a key role in a goal-directed control of perceptual processing (i.e., top-down attention) (Meehan et al., 2017). In contrast, the ventral attention network (VAN) is engaged in the detection of salient and unexpected events (Corbetta and Shulman, 2002). The VAN redirects attention from the present focus to the novel stimulus of interest when very important or noticeable events are detected outside of the present focus of attention, such as a sudden pedestrian crossing, and the VAN is thus considered to be involved in bottom-up attention (Long and Kuhl, 2018). Regarding older drivers, top-down attention has been shown to compensate for reduced road hazard detection due to age-related bottom-up attentional decline, and diminished top-down attention has been shown to lead to vehicle accidents caused by older drivers (Feng et al., 2018).

The right orbital part of the inferior frontal gyrus plays a crucial role in decision making (Besnard et al., 2017; Vaidya and Fellows, 2020). A previous longitudinal neuroimaging study reported that less thinning of the orbitofrontal cortex during adolescence is associated with risky driving behavior in young people (Vijayakumar et al., 2019). Given that a previous driving simulator study reported that the number of violations and accidents was positively correlated with the tendency to make risky decisions in dilemma situations (Ba et al., 2016), structural alterations of this cortical region may have strong effects on unsafe driving behaviors.

The superior frontal sulcus has been repeatedly shown to contribute to working memory in functional MRI studies using the N-back task (Carlson et al., 1998; Heinzel et al., 2016). In our previous study examining the associations between neuropsychological tests and driving ability in healthy older people, lower working memory function was associated with greater risk of unsafe driving (Yamamoto et al., 2020). Furthermore, working memory has been reported to be associated with a driver's ability to retain traffic information for several seconds (Da-Wei et al., 2017) and predict traffic conditions (Jipp and Ackerman, 2016). Considering that our final model identified the cortical regions involved in cognitive functions essential for driving, such as voluntary orienting of attention, decision making, and working memory, as important inputs, the current study provides new insights into the neural basis of driving behavior.

Limitation and Future Works

The results of the current study should be interpreted with caution because of several limitations. First, the number of participants per group was relatively small. In general, small numbers of participants can induce over-fitting. To mitigate this problem, we created a sparse model using the LASSO algorithm. However, it would be optimal to train our classification model with an independent cohort to generalize the model. Future studies with larger samples at multiple sites may be useful for addressing this issue. Second, although we measured on-road driving ability using a data recorder and CCD cameras, we only evaluated one aspect of driving ability. Because we decided not to use other data, including data regarding the pedal position, gaze and footwork, to simplify the classification criteria, it may be valuable to establish a new objective method with which to measure various types of driving ability. For instance, examining driving behaviors when turning right at an intersection or making a lane change could provide useful results. Third, we used a cut-off value of 5 km/h to divide participants into unsafe and safe drivers based on the past findings (Nesamani and Subramanian, 2006; Seers et al., 2015). However, even though using such criteria for the classification, we are not able to completely eliminate the possibility of arbitrariness. Therefore, future studies with large samples are needed to confirm the validity of our classification of safe and unsafe drivers. Finally, previous studies reported that motor dysfunction (Anstey et al., 2017) and hearing impairment (Edwards et al., 2016) are associated with unsafe driving. However, motor and hearing functions were not systematically evaluated in the current study, although the participants were apparently free from these problems. Future studies should be conducted to evaluate motor and hearing functions in more detail.

CONCLUSION

Overall, we built a reliable classification model for identifying the on-road driving ability of healthy older individuals with an accuracy of 87.5%. Five parameters (age and gray matter volume in four cortical regions, including the left superior part of the precentral sulcus, the left sulcus intermedius primus [of Jensen], the right orbital part of the inferior frontal gyrus, and the right superior frontal sulcus), were consistently selected as features for the final classification model. Importantly, the current findings revealed the neural bases of unsafe driving in healthy older people, suggesting that age and gray matter volume data can provide useful information for identifying unsafe drivers, potentially leading to the development of new interventions to prevent fatal traffic accidents.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of the University of Tokyo and Keio University. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YY, BY, JH, MS, and MM designed the study. YY, BY, JH, HY, KN, MY, MK, and KK acquired the data. YY, BY, JH, and RU analyzed and interpreted the results of the data. YY, BY, JH, and MM drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Applying the Inverse Efficiency Score to Visual–Motor Task for Studying Speed-Accuracy Performance While Aging

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Background: The current study examines the relationship between speed and accuracy of performance in a reaction time setting and explores the informative value of the inverse efficiency score (IES) regarding the possibility to reflect age-related cognitive changes.

Objectives: To study the characteristics of speed and accuracy while performing psychophysiological tests throughout the lifespan; to examine the speed-accuracy ratio in age groups and to apply IES to discriminative visual-motor reaction task; and to figure out the predictive potential of psychophysiological tests to identify IES values.

Methods: We utilize nonparametric statistical tests, regression analysis, and supervised machine learning methods.

Results and Conclusion: The examinees under 20 and over 60 years of age share one tendency regarding the speed-accuracy ratio without speed-accuracy trade-off. Both at the time of active developmental changes in adolescence and during ongoing atrophic changes in elderly there is a tendency toward a rise of the number of mistakes while slowing the reaction. In the age range from 20 to 60 the relationship between the speed and accuracy is opposite and speed-accuracy trade-off is present. In our battery, complex visual-motor reaction is the only test with the significant negative association between reaction time and error rate in the subcohort of young and midlife adults taken together. On average, women perform more slowly and accurately than men in the speed-accuracy task, however most of the gender-related differences are insignificant. Using results of other psychophysiological tests, we predicted IES values for the visual-motor reaction with high accuracy ($R^2 = 0.77 \pm 0.08$; mean absolute error / IES range = 3.37%). The regression model shows the best performance in the cognitively preserved population groups of young and middle-aged adults (20–60 years). Because of the individual rate of neurodevelopment in youth and cognitive decline in the elderly, the regression model for these subcohorts has a low predictive performance. IES accounts for different cognitive subdomains and may reflect their

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disproportional changes throughout the lifespan. This encourages us to proceed to explore the combination of executive functioning and psychophysiological test results utilizing machine learning models. The latter can be designed as a reliable computer-aided detector of cognitive changes at early stages.

Keywords: aging, cognitive decline, speed-accuracy trade-off, decision making, error, machine learning, regression model, gender

1. INTRODUCTION

A speed-accuracy trade-off (SAT) in behavioral decisions is a physiological phenomenon that accounts for the adjustment of species to living conditions. SAT is a feature of the individual's psychophysiological status that can dynamically change in a certain range of values under certain conditions, otherwise, it remains stable (Wang et al., 2018). Individuals differ in cognitive styles, and the individual traits of SAT may account for cognitive performance (Jones et al., 2020).

On average, accurate decisions require more time, while fast decisions are usually less accurate. However, in some circumstances, both variants could be an option. Speed-accuracy tactics are known to vary consistently and show a degree of flexibility during task fulfillment. Such individual flexibility in speed-accuracy tactics is likely to be advantageous for animals exposed to fluctuating environments, such as changes in predation threat (Wang et al., 2018). According to the cognitive styles hypothesis, individuals with consistently low levels of activity and higher sensitivity to risk may be expected to take more time but make more accurate decisions than individuals that are more active and less sensitive to risk. Based on this, one can argue that SATs underlie *interindividual differences in cognition* (Jones et al., 2020). This implies that SATs may account for *cognitive styles* which we can discriminate by testing the individuals and estimating their trade-offs. In any test, the examinee has a substantial degree of control over speed or accuracy at which s/he chooses to operate. Yet, the individuals act within the boundaries of personal performance limitations. They may adjust these boundaries continuously depending on error feedback (Fitts, 1966).

The clinical necessity to develop the aforementioned cognitive theories and to measure SAT comes out of the practical importance to estimate psychophysiological status by providing clearly stated metrics of its current condition and changes.

Abbreviations: AC, asymmetry coefficient; AST, attention study technique; CVMR, complex visual-motor reaction; DMT, decision making time; ER, error rate; IES, inverse efficiency score; IQR, interquartile range; IRT, interference resilience technique; LOWESS, locally weighted scatterplot smoothing; MAE, mean absolute error; ML, machine learning; OLS, ordinary least squares; POBA, psychophysiological outcomes of brain atrophy (the name of a project and the dataset obtained); PS, psychophysiological status; PT, psychophysiological test; RMO, reaction to a moving object; RMSE, root mean squared error; RT, reaction time; SAT, speed-accuracy trade-off; SVMR, simple visual-motor reaction; TRVI, the time delay in responding to the targeted stimulus because of visual interfering objects.

1.1. Speed and Accuracy Performance During Development and Atrophy

1.1.1. The Impact of Neurodevelopment on Speed and Accuracy

Even within a narrow 5–7 years of age participants group, researchers found that *older children are faster and more accurate* (Torpey et al., 2012). In this study, associations between the gender of the child and the behavioral modes suggested that girls were more cautious than boys as found in other age groups.

1.1.2. Age-Related Changes in Speed and Accuracy

Many findings indicate a consistent pattern of increased reaction time (RT) with age in both genders in a variety of tasks. Fozard et al. (1994) conducted a longitudinal study of aging with adult volunteers 17–104 years of age. They estimated a consistent slowing down of auditory reaction which starts at about the age of 20 and increases at a rate of approximately 0.5 ms/yr for simple reaction and 1.6 ms/yr for the disjunctive reaction. In this observation the number of errors also increased throughout the lifespan, *making a tradeoff of accuracy for faster responses unlikely*.

Facts suggest a possible *impairment of response inhibition* in old age. As an outcome of this, the elderly may experience difficulty with sustaining attention to the stimuli exclusively. Presumably, this is caused by a decreased ability to inhibit irrelevant thoughts (Arbuckle and Gold, 1993). The individual features and the age-related changes in visual-attention control may also account for different cognitive performance. This may happen at least for two reasons. These are either a decline in overall bottom-up sensory input, or decrease in the differentiation of top-down goal-directed representations (Heitz and Engle, 2007; Li et al., 2013).

1.2. A Statistical Approach to the Problem

A number of studies have developed various models to account for the speed-accuracy payoff (Thompson, 2007). This is based on the fact that a part of the choice-reaction time is devoted to decision making and processes that are analogous to those employed in the simple sensorimotor reaction (Fitts, 1966). *The random walk model* initially suggested by Stone (Stone, 1960) is one of the simplest to make time series forecasting. It possesses enough structure to predict accuracy and latency results. The random walk model for two choice reaction times assumes that there is an information accumulation over the course of perceptual decision-making. In other words, evidence in favor of these responses is accumulated gradually over time until the evidence favoring one over the other exceeds some preset criterion, at which time the favored response is emitted

(Ashby, 1983). The random walk model is a sequential model, i.e., it assumes the incremental evidence accumulation, i.e., faster responses entail less accumulated evidence, and hence less informed decisions (Heitz, 2014). As an alternative to this, Ollman explained test performance with *a mixture model*. It is a mixture of dichotomous states: fast guesses and slow controlled decisions. The most obvious is that error RT is in average faster than correct RT (Ollman, 1966). The fact that error RT is sometimes faster and sometimes slower than correct RT is problematic for mixture models (Heitz, 2014).

The correct-to-wrong responses ratio reflects the accuracy and accounts for decision processes and the level of education of the examinee. The reaction time (RT) length is determined by cognitive processes and by an individual's use of the feedback information. However, the performance metrics (e.g., RT, ER) may deteriorate if the examinee is set for speed vs. accuracy, or vice versa (Fitts, 1966). The reason for this lies in the ambiguous instruction to maintain both high accuracy and fast RT (Heitz, 2014). For natural reasons emphasis on speed decreases mean reaction time but increases errors. EEG and fMRI studies evidenced that SAT manipulations affect more than decision process (particularly, decision threshold), they alter decision post-processing as well (Nieuwenhuis et al., 2001; Bogacz et al., 2006; Heitz, 2014).

Formerly, authors tried to compare velocity and accuracy characteristics of test performance when subjects are set for speed or correctness. They figured out that error rate may become very small in circumstances where the penalty for errors is sufficiently high, but it never becomes zero as long as the speed of response is given any importance at all (Hick, 1952; Hyman, 1953). Howell and Kreidler (1963) carried out a true SAT experiment in which different groups of participants were asked to favor fast or accurate over fast and accurate responding. With this methodology one can estimate the time cost of an error. SAT is an exact answer to the issue of the time cost of an error. Fitts (1966) pointed out that when the payoff for speed is reasonably high it may induce errors at any level of intelligence or attention. Hence, the occurrence of errors should not be considered as a qualitative change in performance. In such a way, the attempts to induce either "errorless" or accelerated performance are low-informative and their interpretation seems to be challenging.

1.3. Speed-Accuracy Estimates Inside Standard Batteries of Psychophysiological and EF Tests

Psychophysiological tests (PT) describe cognitive functioning in terms of domains of functioning. As the cognitive functions are closely linked, it is a challenging task to interpret the changes that may account for mutual compensation (like, SAT). The compensation accounts for the different rate of decline within different cognitive domains. Worsening of diverse performance metrics may start at any age and progress independently. For example, playing tennis preserves high accuracy in coincidence timing performance (Lobjois et al., 2006). Strategic compensation may allow older adults to execute *decision making* at high levels of accuracy (Fechner et al., 2019). The age-related changes in speed and accuracy performance are common results of cognitive retardation (Thapar et al., 2003), however the ratio between them is not studied well-throughout the lifespan.

Clinically, cognitive functioning (e.g., inhibition) is often assessed with the *Stroop color-naming task* different versions of which match the issue of the SAT. In a classical variant of the test, the individual has to inhibit an automatic reading response and to produce the more effortful color-naming task. *The extra time* required to name colors in the interference task compared to the control task (B-A) represents the interference effect (*interference score*). In parallel to this, the *error scores* are recorded. The dualism of executive functions provoked researchers to elaborate a new neuropsychological test that would assess concurrently both inhibition and switching. Such was *Stroop switching test*. Additionally to the classic interference condition (the situation when the word meaning and the ink color doesn't fit), it included switching in-between tasks. The possible clinical application of the idea is to reveal early executive dysfunction that remains undetected with standard neuropsychological tests. So, as opposed to other tests, the compensation strategies do not hinder the early stages of executive dysfunctioning in Stroop switching test. By following the same pipeline, Belghali and Decker (2019) created a modified Stroop switching test version named Stroop switching card test. It encompasses additional conditions and metrics to appraise some more cognitive features so that the global and local variables of the novel test (e.g., the total number of errors performed) reflect the overall individual's executive functioning. The global metrics of performance in the test are the overall time spent on the test and the number of errors done.

$$IES = \frac{TIME}{1 - ERROR} \tag{1}$$

To study SAT with the tests, researchers combine speed and accuracy into a single dependent variable called the inverse efficiency score (IES). The variable derives from the mean RT and ER. From Equation (1), IES is expressed in milliseconds as well as RT, however, it indicates roughly the time spent for correct responses. When there is a trade-off between speed and accuracy, the IES effect will compensate for the differences in the percentage of incorrect responses.

2. OBJECTIVES

To get insight as to whether speed-accuracy ratio can serve as a potential biomarker of individual cognitive status while aging we address the following sub-objectives:

1. To study the characteristics of speed and accuracy while performing psychophysiological tests throughout the lifespan.
2. To study the association of the speed-accuracy ratios with the age and cognitive subdomains.
3. To examine the speed-accuracy ratio in age groups and with regard to the gender.
4. To figure out the predictive potential of PTs to identify the values of inverse efficiency scores.

3. MATERIALS AND METHODS

3.1. The Used Battery of Tests

The battery of neurophysiological tasks we used fits the idea of our study: it contains PTs with both accuracy and time performance metrics. It has a well-considered structure and reflects a set of cognitive subdomains that underlie goal-targeted behavior. The examinees were paid neither for taking part in the study nor for the achieved results (e.g., good timing or error-less performance) otherwise this would be a limiting non-physiological factor that could spoil outcomes of the research. Structurally, the dataset consists of a list of deidentified subject records, a patient per row, and stored in the comma-separated value format files.

3.2. "Psychophysiological Outcomes of Brain Atrophy" Dataset

The dataset is named after the title of the project Psychophysiological Outcomes of Brain Atrophy (POBA). POBA consists of about 100 features reflecting the overall psychophysiological status of examinees. The battery of tests covers diverse aspects of cognitive functioning, both high-level and basic-level ones. It includes 231 cases of MRI examination and PT of people of different ages (4–83 yo). Written patient's consent or parental consent with assent from minors for being tested and scanned was obtained in each case. All the examinees are either patients who suffer from periodic headaches and are anxious about having organic brain pathology or healthy participants examined at the beginning of their professional sports career. The exclusion criteria were as follows: organic brain pathology, mental disorders, injury to the head. The dataset is provided on demand (See section 7). The following tests' form POBA dataset:

1. Simple visual-motor reaction (SVMR). SVMR is the test with the only type of stimulus requiring one and the same response. RT, deemed as the time elapsing between the onset of the stimulus and the initiation of the response, is the major dependent variable. SVMR_mean (ms) is the mean value calculated out of over 30 (SVMR_trialsNo) subsequent episodes of testing with unequal intervals of time between them. SVMR_mean (ms) is the value calculated out of over 30 subsequent episodes of testing (SVMR_trialsNo) with unequal intervals of time between them.

The median (SVMR_median) and the mode (SVMR_mode) values also describe the sample. The measures of how the length of the reaction are scattered in time are the standard deviation (SVMR_variance), the kurtosis (SVMR_kurtosis), the asymmetry (SVMR_ass), the quartile values (SVMR_q25, SVMR_q75), and the half-size interquartile range (SVMR_(q75-q25)/2).

From the physiologic point of view, they may serve as markers of how stable the reaction is in time. Another dependent variable is the number of mistakes made by the examinee (SVMR_mistakes). The mistakes fall into two categories. These are 1. missing the targeted events (SVMR_passes) and 2. preliminary responding (SVMR_falstart). Another dependent variable derived from the test is the Whipple's accuracy coefficient (SVMR_acc_coef). It shows the ratio of errors to correct responses. The lower the indicator is the higher is the performance accuracy. It reflects how stable the attention is and how balanced nervous processes are. In other words, it is a marker of the balance of the excitatory and inhibitory processes.

To assess the sensorimotor response the system also calculates the following indices by Loskutova: system functional level (SVMR_sfl), the reaction stability (SVMR_rs), and the functional ability level (SVMR_fal) (TD, 1975). SVMR_sfl showes the current functional state of the central nervous system at the time of examination, including the rise up of the fatigue. SVMR_rs reflects the stability of the nervous system functioning. The bigger it is, the less the individual's test results are scattered in the time length. The functional ability level (SVMR_fal) is the most valuable index. It reveals the ability of the subject to form a functional system adequate to the task and sustain it for a long-time period. This means, the variable describes the capacity to adjust.

With SVMR time, one can assess the mobility of nervous processes: the shorter RT is, the higher the more mobile the nervous processes are. The variance of the variable (SVMR_variance) depicts the balance of the processes, i.e., the smaller the standard deviation is, the more balanced the nervous system is. SVMR_mean value under 177 ms accounts for the pronounced mobility of nervous processes. Its value of 177–200 ms is a characteristic of a mobile type of nervous processes. With 200–210 ms SVMR_mean length the average type of nervous processes is diagnosed. SVMR_mean value of 210–233 ms is typical for the inertia of nervous processes. Finally, if the reaction lasts more than 233 ms, the pronounced inertia of nervous processes is reported.

Studying the dynamics of RT indicators throughout a day is a way to estimate the strength of the nervous system. With a strong (steady, balanced) nervous system, RT does not change significantly during the day and within the framework of one examination.

2. Complex visual-motor reaction (CVMR) is a variant of choice reaction test. A visual stimulus is presented to a subject whose task is to make a motor response quickly and accurately. We used the go/no-go test in which the examinee is asked to push the response button at green indicator light. In contrast, when the indicator lights up with red color no motor response is required. The system records the time elapsed between the onset of a stimulus and a response to. CVMR_mean is the mean length of response time calculated after 30 subsequent presentations of the triggering stimulus. CVMR_mistakes is the number of the fault responses. In the data analysis we use a derivative variable called error rate (ER) (see Equation 3). As in CVMR, we implemented the error rate derivative variables in other tests as well (e.g., SVMR, etc.). There is one more type of the mistakes in the complex reaction compared to the simple one. It is a false reaction to the triggering stimulus of the wrong color, i.e., the responding to the red splash rather than to the green one (CVMR_false_reaction). Similarly to the simple reaction, the complex one also has such dependent variables as CVMR_median, CVMR_mode, CVMR_variance, CVMR_kurtosis, CVMR_ass, CVMR_q25, CVMR_q75, CVMR_passes, CVMR_falstart, CVMR_acc_coef.

Decision-making time (DMT) is the difference between the length of the simple visual-motor reaction and the complex one (see Equation 2). DMT reflects the time cost of the response selection. At the time of making the choice the individual affiliates the cognitive subdomains of switching and inhibition, i.e., the person inhibits prepotent responses and shifts between tasks.

3. Attention study technique (AST) is the test in which the examinee is asked to respond to visual stimuli that flick one after another 30 times in diverse parts of the computer screen. Instead of targeting a single point like in the simple visual-motor reaction, the participant is to concentrate attention on the entire PC screen and respond as soon as possible by allocating the same motor reaction as recently.

Reasonably, the dependent variables of AST are similar to that ones in the simple motor-visual reaction time. These are RT (AST_mean, AST_median, AST_mode), the standard deviation (AST_variance), the kurtosis (AST_kurtosis), the asymmetry (AST_ass), the total number of errors with the number of missed stimuli and preliminary responding inside of them (AST_mistakes, AST_delays, and AST_falstart correspondently), the Whipple's accuracy coefficient (AST_acc_coef), and the indices by Loskutova the system functional level (AST_sfl), the reaction stability (AST_rs), the functional ability level (AST_fal).

Additionally, the system provides two estimators that are specific to AST test. These are the attention stability (AST_stability) and the concentration of attention (AST_concentration).

4. Interference resilience technique (IRT) is a bit more complex since the triggering stimuli are obscured with additional interfering objects that appear on the PC screen overlapping the targeted ones (see **Figure 1**). As the task paradigm is close to the attention study technique, the list of the dependent variables is almost similar.

It includes RT (IRT_mean, IRT_median, IRT_mode), the standard deviation (IRT_variance), the kurtosis (IRT_kurtosis), the asymmetry (IRT_ass), the total number of errors with the number of missed stimuli and preliminary responding inside of them (IRT_mistakes, IRT_delays, and IRT_falstart correspondently), the Whipple's accuracy coefficient (IRT_acc_coef), and the indices by Loskutova the system functional level (IRT_sfl), the reaction stability (IRT_rs), the functional ability level (IRT_fal).

In analogy to DMT, we calculate the time delay in responding to the targeted stimulus because of visual interfering objects (TRVI) as in Formula 4 by substituting of the mean RT without interfering objects (AST_mean) from the study technique with them (IRT_mean).

5. Reaction to a moving object technique (RMO) reflects either the balance of two opposite processes in the central nervous system (e.g., excitation and inhibition) or the predominance of any of them. At the time of the task a circle appears on the screen with two marks of red and green color arranged radially. It is gradually filled quickly with yellow color, from some starting point to the finishing line in a clockwise direction, like, in **Figure 2**.

The examinee should respond when the yellow sector passes through the red finishing line. There is a total number of over 30 trials (RMO_trialsNo). The result is processed as a mean value (RMO_mean) of positive (the time delays) and negative values (the premature responses). When the RMO_mean value is negative, it indicates the predominance of excitation of the central nervous system. If being positive, RMO_mean reveals the predominance of inhibition of the central nervous system. The tester counts the number of responses that were accurate in time (RMO_acc), the delayed ones (RMO_delays), and the fault starts (RMO_falstart). Also, the application sums up the time of them (RMO_positiveSum, RMO_delaysTotalTime, RMO_falstartTotalTime). All the trials disregarding their accuracy pertain to any of two classes, i.e., the "positive" trials when the response comes at any time after the

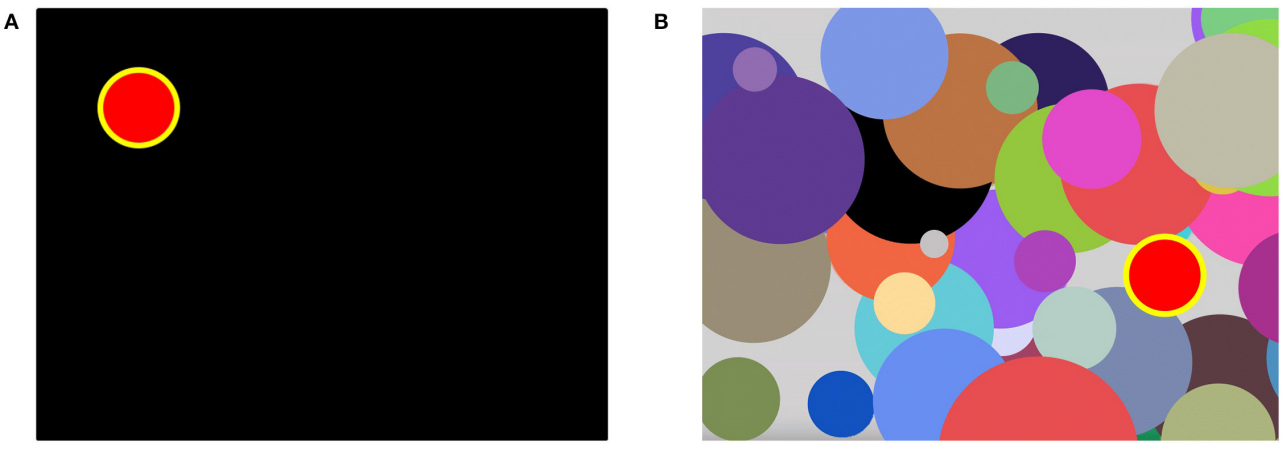

FIGURE 1 | The appearance of the computer screen while testing with attention study technique **(A)** and with interference resilience technique **(B)**. In **(A)**, the stimuli appear sequentially one after another in different parts of the screen. In **(B)**, the interfering visual stimuli pad the screen and complicate the response to specific triggering stimuli.

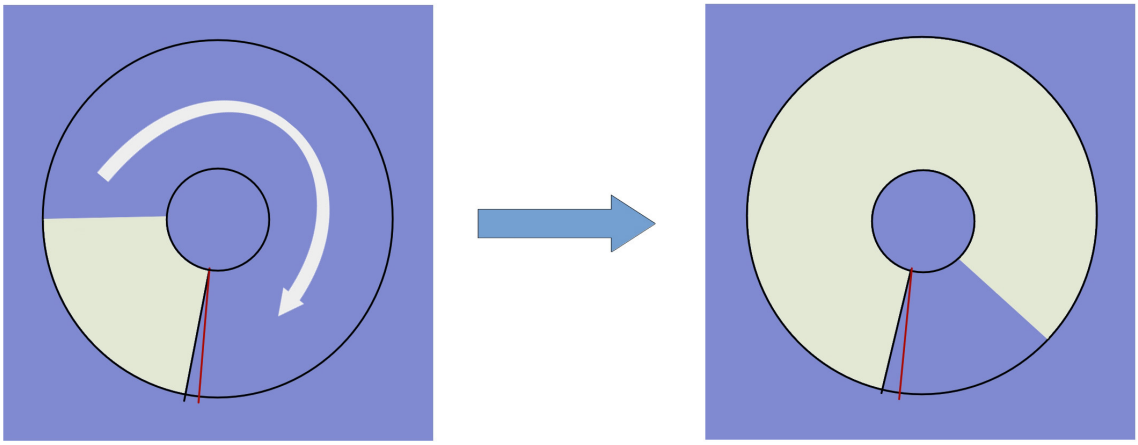

FIGURE 2 | Testing reaction to a moving object: the circle is gradually colored yellow clockwise. The examinee is supposed to respond at the red finishing mark.

targeted event and the "negative" ones when the response comes before the event. There are summary counters for the number of positive trials (RMO_positiveCount) and the number of negative ones (RMO_negativeCount).

Like the tests mentioned above, RMO also has such dependent variables as RMO_variance, RMO_median, RMO_mode, RMO_ass, RMO_kurtosis, RMO_q25, RMO_q75. Additionally, there are such metrics as entropy (RMO_entropy), the number of responses

6. Wrist dynamometry is a way of evaluating the maximum muscular strength of the right (*WDR_MMS*) and the left hands (*WDL_MMS*). *Asymmetry coefficient (AC)* is calculated from Equation (5).

We calculate the inverse efficiency scores (SVMR_IES, CVMR_IES, AST_IES, IRT_IES) as Formulas 6-9 state.

$$DMT = CVMR\_mean - SVMR\_mean \tag{2}$$

$$CVMR\_error\_rate = \frac{CVMT\_mistakes}{CVMT\_trials} \tag{3}$$

$$TRVI = IRT\_mean - AST\_mean \tag{4}$$

$$AC = \frac{WDR\_MMS}{WDL\_MMS} \tag{5}$$

$$SVMR\_IES = \frac{SVMR\_mean}{1 - SVMR\_error\_rate} \tag{6}$$

$$CVMR\_IES = \frac{CVMR\_mean}{1 - CVMR\_error\_rate} \tag{7}$$

$$AST\_IES = \frac{AST\_mean}{1 - AST\_error\_rate} \tag{8}$$

$$IRT\_IES = \frac{IRT\_mean}{1 - IRT\_error\_rate} \tag{9}$$

3.3. Age Groups

For the comparison of age cohorts, we added attribute "Groups" to the POBA dataset. The range of years corresponding to age groups is as follow: Adolescent age $\in [0, 20)$, Young adults age $\in [20, 40)$, Midlife adults age $\in [40, 60)$ and Older adults age is ≥ 60.

3.4. The Methodology of the Study

To address the first objective, we used both the descriptive statistics and the machine learning approach.

As the variables of our dataset had non-normal distribution, we utilized non-parametric tests for the analysis. In age groups, the relationships between continuous features were assessed with Kruskal-Wallis test.

Also we analyzed the charts that describe age-related changes of the mean RT and accuracy. To build the trendlines we used non-linear Locally Weighted Scatterplot Smoothing (LOEWSS), which is a non-parametric regression method that combines multiple regression models in a k-nearest-neighbor-based meta-model.

To address the second objective we proposed new psychophysiological indices equivalent to inverse efficiency score in EF tests. As the paradigm of the psychophysiological tests we worked with fitted the idea of SAT, we came up with the indices equivalent to the inverse efficiency score in EF tests. These are *SVMR_IES*, *CVMR_IES*, *AST_IES*, and *IRT_IES* (see Formulas 6–9). RMO test results include variables that estimate time and mistakes done. However, in this test, the time variables are the additional estimators of the reaction accuracy (e.g., the delayed reaction or the proactive one) rather than RT. So, the test doesn't fit the concept of SAT, and it cannot provide researchers with an efficiency score.

In the first part of objective number two, we inspected possible associations between the age and the newly proposed scores. For this, we built the ordinary least squares (OLS) regression trendlines with a 95% confidence interval. By using Ridge regression we approximated the parameters of the

model (e.g., the slope and the intercept) with the polynomial function of degree one and assessed the performance of the models.

To compare the lifelong dynamics of the IES scores for different tests (e.g., CVMR, SVMR, AST, IRT) we tested the linear models for statistically significant differences between the slopes. For this we used statistical hypothesis tests, specifically *t*-test.

To improve the reproducibility and interpretability of the age-related trends we also did sensitivity analyses, in which the RT and error outliers were removed.

Working on the second part of objective two, we assessed the relationship between age, tests results that reflect diverse cognitive subdomains and the proposed scores. To do so, we calculated Spearman rank correlation coefficients and checked the associations of variables for the significancy.

To address the third objective we inspected possible associations between the mean RT and the overall number of mistakes in age and gender groups. We analyzed the OLS regression trendlines with a 95% confidence interval.

We studied whether the age and gender affected the impact of the RT change on the error rate, i.e., if there is an interaction effect. For this we tested our data for statistically significant differences between the slopes.

The second part of the third objective was to find, whether the gender influences the dynamics of age-related changes in RT, efficiency score, mistakes. The statistical approach to this objective was the same as mentioned above. For the gender comparison, we removed outliers from the cohorts of females and males separately and then plotted the trendlines. We assessed the relationships between continuous features with Mann-Whitney *U*-test.

In the fourth objective we figured out if IES provides a summary of the findings obtained while testing individuals. This could give the insight to what extent the novel inverse efficiency score reflects the overall psychophysiological status (PS).

To evaluate the prediction potential of the PS estimates to reflect the *CVMR_IES* value, we used the following predictors: "DMT," "SVMR_mean," "SVMR_mistakes," "AST_mean," "AST_mistakes," "IRT_mean," "IRT_mistakes," "TRVI," "RMO_mean," "RMO_mistakes," "WDL_MMS," "WDR_MMS," "AC," "age."

For addressing the objectives of the study we utilized supervised machine learning methods, namely regression models. We did this because the predicted values were continuous. Doing this, we used a common approach to data analysis. First, we chose the set of predictors and did a data standardization (removing the mean and scaling to unit variance) as usually required by many machine learning estimators. Then the data were shuffled and fed to the ML regression models in a 10-fold cross-validation manner. In this study, we used such regression algorithms as Lasso, Support Vector Regression with radial basis function kernel, K-nearest neighbors, Gradient Boosting, AdaBoost, and Random Forest. To evaluate the performance of regressors, mean absolute error (MAE), root mean squared error (RMSE), and coefficient of determination (R^2) performance metrics were used.

3.5. Hardware and Software Used

All the experiments were conducted with the Linux Ubuntu 18.04 workstation with 24 CPU cores and two NVIDIA GeForce GTX 1080 Ti GPU with 11 GB GDDR5X memory each using programming language Python, and its libraries for Data Processing, ML and Data visualization, such as scikit-learn, NumPy, Pandas, Matplotlib, Seaborn, Plotly.

4. RESULTS

4.1. The Characteristics of Speed and Accuracy While Performing PT

Table 1 presents the results of testing between-groups differences in psychophysiological variables that reflect reaction speed, accuracy, their ratio, and the information speed processing (e.g., DMT, TRVI). Both DMT and TRVI are the time of inhibition of an automatized action and task switching in the standardized tests. We assume that adolescents experience more difficulties with the concentration of attention, and this impacts the performance of the attention-related tasks, particularly *AST* and *IRT*.

The results of Kruskal-Wallis test state that median values of the variables mentioned above differ significantly in the age groups. We also noticed that all groups are drawn from people with different median values of *AC* and *DMT* which is a marker of the information processing speed. However, there was no significant differences in the median values of *TRVI* between the groups ($p = 0.148$). The supposed reason for this is that TRVI values were highly scattered in the time scale in the group of adolescents (59.85 ± 60.46 ms). The values of the DMT are heteroscedastic throughout the lifespan, showing the high variance in adolescent group (63.08 ± 52.97 ms).

Figure 3 describes age-related changes of mean RT and accuracy. To build the trendlines we used non-linear Locally Weighted Scatterplot Smoothing (LOWESS), which is a non-parametric regression method that combines multiple regression models in a k-nearest-neighbor-based meta-model.

4.2. The Association of Age and Cognitive Subdomains With the Novel IES From PT

4.2.1. The Possible Associations Between the Age and the Newly Proposed Scores

Figure 4A shows OLS regression trendlines with a 95% confidence interval. The trendlines reflect changes of SVMR_IES, CVMR_IES, AST_IES, IRT_IES throughout the lifespan. **Figure 4B** illustrates the age-related trends of the variables after the sensitivity analyses, in which the RT and error outliers are removed. It is worth mentioning that sample size was reduced to 161 points in average after the removal of the subjects which outstand the range from 15 to 85 percentiles. **Table 2** presents the performance of the OLS regression models so that one can estimate how the data reproducibility improved with the outliers being removed.

To compare the lifelong dynamics of the IES scores from different tests (e.g., CVMR, SVMR, AST, IRT) we analyzed

TABLE 1 | Tests performance in age groups.

Variable	Total		Adolescent	Young adults	Midlife adults	Older adults	*p*-value
	Median	IQR					
N	231		48 (20.78%)	64 (27.71%)	64 27.71%)	55 (23.81%)	
Female	134 (58.01%)		19 (39.58%)*	36 (56.25%)	39 (60.94%)	40 (72.73%)*	**<0.0078**
Male	97 (41.99%)		29 (60.42%)*	28 (43.75%)	25 (39.06%)	15 (27.27%)*	
Age	40.96	24.87–59.76	11.52 ± 3.37*	29.93 ± 5.09*	49.9 ± 6.09*	68.09 ± 6.61*	**<0.001**
DMT	93.84	63.6–122.43	63.08±52.97*	99.88±48.64	96.32 ± 51.65	101.97 ± 57.81	**<0.0006**
TRVI	61.9	34.7–96.0	59.85 ± 60.46	47.0 ± 39.25*	66.55 ± 45.34	81.4 ± 74.18	0.148
AC	1.08	1.01–1.19	1.13 ± 0.25*	1.06 ± 0.14	1.07 ± 0.2	1.09 ± 0.17	**<0.0079**
SVMR_mean	245.52	219.63–285.83	263.88 ± 70.91*	215.22 ± 28.92*	242.02 ± 55.48	282.63 ± 53.75*	**<0.001**
CVMR_mean	350.75	307.45–395.57	350.82 ± 107.74	320.24 ± 56.55*	354.58 ± 65.15	386.15 ± 71.9*	**<0.001**
AST_mean	343.4	311.05–412.35	343.7 ± 58.96	309.95 ± 46.58*	339.9 ± 61.86	416.0 ± 60.93*	**<0.001**
IRT_mean	413.8	368.15–471.85	405.25 ± 84.98	369.1 ± 46.63*	417.6 ± 70.65	473.8 ± 75.08*	**<0.001**
RMO_mean	7.6	−18.5–31.35	−0.7 ± 69.28	3.6 ± 54.25	22.8 ± 104.22*	8.9 ± 75.59	**<0.0065**
SVMR_variance	57.7	41.09–80.82	63.7 ± 73.36	42.86 ± 22.39*	56.64 ± 36.54	67.45 ± 42.92*	**<0.001**
CVMR_variance	91.81	70.7–118.64	97.72 ± 94.58	79.53 ± 80.43*	87.19 ± 30.46	117.08 ± 74.86*	**<0.001**
AST_variance	72	53.55–115.6	70.85 ± 57.47	56.65 ± 38.28*	74.4 ± 45.85	112.2 ± 62.13*	**<0.001**
IRT_variance	103.5	76.75—156.85	133.8 ± 78.28*	78.7 ± 40.12*	94.95 ± 49.33	150.3 ± 70.46*	**<0.001**
RMO_variance	140.3	84.7–224.35	142.1 ± 103.5	101.4 ± 67.33*	130.35 ± 93.83	216.7 ± 105.18*	**<0.001**
SVMR_mistakes	0	0.0–2.0	1.5 ± 3.83*	0.0 ± 1.32*	0.0 ± 1.11*	1.0 ± 1.54*	**<0.001**
CVMR_mistakes	2	1.0–4.0	3.0 ± 2.45*	2.0 ± 2.81*	2.0 ± 1.75*	3.0 ± 2.26*	**<0.0003**
AST_mistakes	1	0.0–2.0	0.0 ± 2.09	0.0 ± 1.16*	1.0 ± 2.28	2.0 ± 3.33*	**<0.001**
IRT_mistakes	3	1.0—6.0	4.0 ± 3.21	2.0 ± 2.35*	3.0 ± 3.74	6.0 ± 4.09*	**<0.001**
RMO_mistakes	22	18.0–24.0	20.5 ± 5.22	18.5 ± 4.14*	22.0 ± 3.82*	24.0 ± 3.34*	**<0.001**
SVMR_IES	253.7	224.94–304.73	282.32 ± 236.3*	223.14 ± 32.9*	246.86 ± 59.02	294.43 ± 64.35*	**<0.001**
CVMR_IES	382.34	336.52–448.65	383.59 ± 143.57	346.08 ± 81.36*	382.92 ± 66.29	447.78 ± 95.44*	**<0.001**
AST_IES	357.1	318.6–443.48	353.3 ± 88.68	317.8 ± 57.35*	351.86 ± 96.73	448.71 ± 141.53*	**<0.001**
IRT_IES	458.25	398.08–591.65	454.08 ± 187.52	394.6 ± 70.9*	469.82 ± 169.36	592.44 ± 221.69*	**<0.001**

**If the variance of a variable differs significantly (p > 0.05) compared to other cases taken together, its Median ± SD is marked with an asterisk. The significant differences between cohorts are marked in bold.*

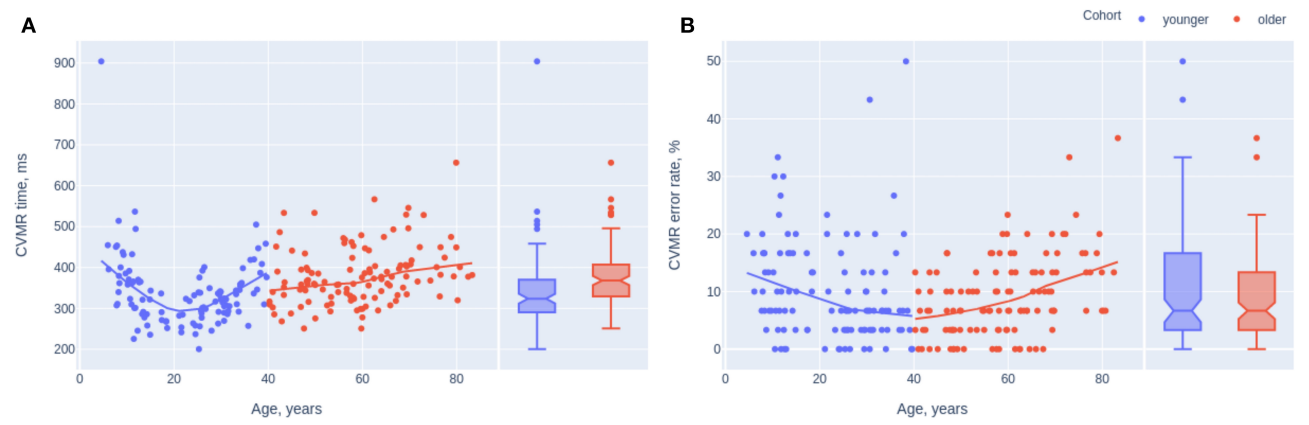

FIGURE 3 | Scatterplots and trendlines presenting the dynamics of reaction time **(A)** and inaccuracy **(B)** while completing CVMR test. The participants below 40 years of age are marked in blue, above 40–in red.

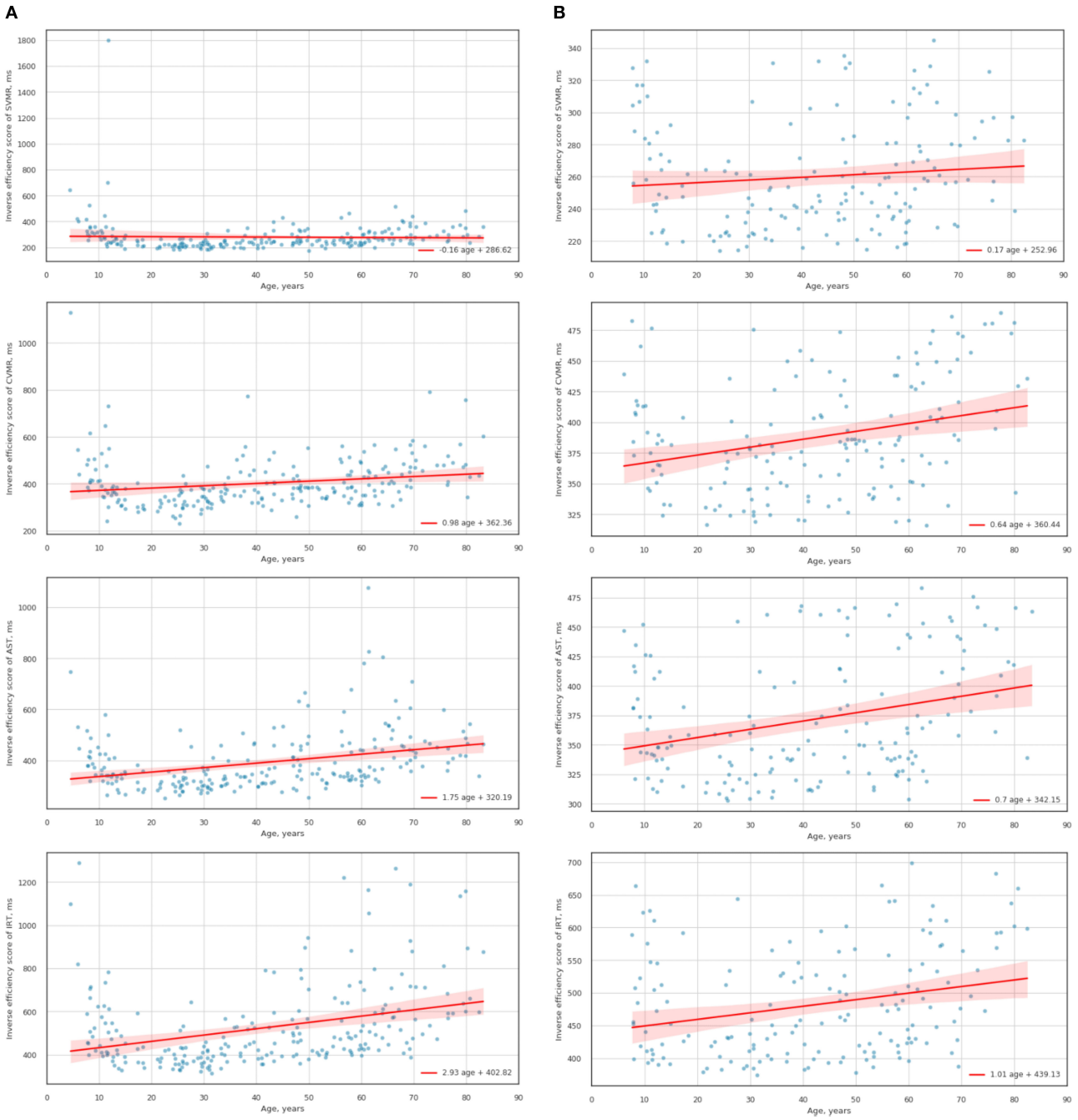

FIGURE 4 | Scatterplots and trendlines of the lifelong changes in IES scores for psychophysiological tests (e.g., CVMR, SVMR, AST, IRT) before **(A)** and after removing the outliers **(B)**.

the linear models. Specifically, we wanted to figure out if there is statistically significant differences between the variables' growth rate(slopes). For this we used statistical hypothesis tests, specifically *t*-test.

4.2.2. The Association of the Novel IES for PT With the Cognitive Estimates

Table 3 reflects relationships between age, tests' results that reflect diverse cognitive subdomains and the proposed scores.

In **Table 4**, there are Spearman rank correlation coefficients and the p-values that reflect the significancy of the associations of age with time estimates of switching conditions (e.g., DMT, TRVI).

4.3. Relationship Between the Mean Reaction Time and the Overall Number of Mistakes in Subcohorts

4.3.1. The Age-Related Trends in the Reaction Time, Efficiency Score, and Mistakes

To study the relationship between the mean reaction time and the overall number of mistakes in age-groups, we analyzed the OLS regression trendlines with a 95% confidence interval. **Figure 5A** presents the whole dataset whereas **Figure 5B** shows scatter plots with trends for the same groups after the sensitivity analysis. The removal of outliers for both variables (e.g., the reaction time and the number of mistakes) reduced the sample size down considerably to 79 cases. However, the observed tendencies remain roughly the same as before. With the reduced sample size it is hard to extrapolate them to the global population. Adolescents and older adults shared one tendency: the number of mistakes increased when the individual boosted the task completion. Young and midlife adults revealed the opposite tendency, i.e., the accuracy of their responses was better when the reaction was quick rather than when it was slow. Subsequently, we removed the outliers from CVMR time and CVMR error rate values which reduced the study sample size from 231 to 79 points. Nonetheless, the sample size reduction preserved the tendency when the people of the age range from 20 to 60 years (young and the midlife adults) had one type of time-to-accuracy association whilst adolescents and older adults had the opposite type. To check if there is an association between RT and age, ER we calculated Spearman rank correlation coefficients presented in **Table 5**.

4.3.2. The Gender-Related Traits in the Reaction Time, Efficiency Score, and Mistakes

Table 6 shows the descriptive statistics on the tests performance in both genders. At the bottom of the table it is well seen that females and males were distributed unequally across the age groups. There were significantly more boys rather than

TABLE 4 | The association of the age and the time estimate of switching condition.

	Age	
	Spearman	***p*-value**
DMT	0.204	**0.002**
TRVI	0.077	0.242

TABLE 2 | Characteristics of the linear models of the lifelong dynamics of IES for psychophysiological tests.

	Whole dataset				Without outliers			
	SVMR_IES	***CVMR_IES***	***AST_IES***	***IRT_IES***	***SVMR_IES***	***CVMR_IES***	***AST_IES***	***IRT_IES***
Slope	−0.159067	0.98327	1.74729	2.92665	0.16592	0.643205	0.7016	1.006818
Intercept	286.618	362.364	320.187	402.8234	252.9606	360.436189	342.1493	439.1339
R^2	0.000741	0.040935	0.10976	0.108747	0.010645	0.083782	0.088361	0.06731
MAE	62.314987	69.338548	75.4813	128.482125	26.9876	35.992386	41.237137	66.063
MSE	15540.02	10314.252	11275.711	31964.0431	1075.506	1936.7576	2358.138119	6153.55

TABLE 3 | Association of the IES scores with the age and other psychophysiological tests results.

	SVMR_IES		***CVMR_IES***		***AST_IES***		***IRT_IES***	
	Spearman	***p*-value**	**Spearman**	***p*-value**	**Spearman**	***p*-value**	**Spearman**	***p*-value**
Age	0.178231	**0.00661**	0.309150	**<0.001**	0.375836	**<0.001**	0.385219	**<0.001**
Error Rate, %	0.431228	**<0.001**	0.300759	**<0.001**	0.507613	**<0.001**	0.446721	**<0.001**
DMT	−0.102335	0.12	0.463131	**<0.001**	0.121777	0.06	0.145347	**0.03**
TRVI	0.209057	**<0.001**	0.173764	**0.01**	−0.046755	0.48	0.461158	**<0.001**
AC	0.093113	0.15837	0.065160	0.32	0.141051	**0.03**	0.129164	*0.05*
RMO_mean	0.051543	0.44	0.079591	0.23	0.119649	0.07	0.053657	0.42
RMO_variance	0.587037	**<0.001**	0.548907	**<0.001**	0.581189	**<0.001**	0.562043	**0.01**
RMO_mistakes	0.543671	**<0.001**	0.517836	**<0.001**	0.533907	**<0.001**	0.590327	**<0.001**
SVMR_variance	0.744956	**<0.001**	0.700105	**<0.001**	0.507649	**<0.001**	0.514861	**<0.001**
CVMR_variance	0.490674	**<0.001**	0.674550	**<0.001**	0.440929	**<0.001**	0.446280	**<0.001**
AST_variance	0.567648	**<0.001**	0.541902	**<0.001**	0.833564	**<0.001**	0.651350	**<0.001**
IRT_variance	0.517957	**<0.001**	0.503068	**<0.001**	0.508932	**<0.001**	0.745864	**<0.001**

The significant associations between features are marked in bold.

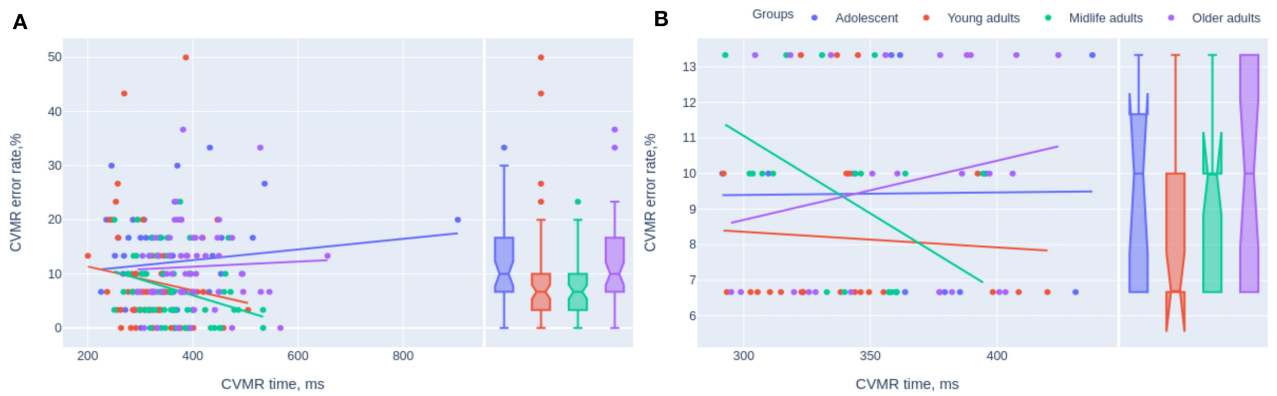

FIGURE 5 | Scatterplots of the mean reaction time and the error rate in CVMR test. The trendlines present the tendencies for different age cohorts before **(A)** and after removing the outliers **(B)**.

TABLE 5 | The association of the reaction time with the age and the percentage of the correct responses.

	SVMR_mean		CVMR_mean		AST_mean		IRT_mean	
	Spearman	***p*-value**	**Spearman**	***p*-value**	**Spearman**	***p*-value**	**Spearman**	***p*-value**
Age	0.219941	**0.00076**	0.317778	**<0.001**	0.368608	**<0.001**	0.382319	**<0.001**
Error rate,%	0.232336	**0.00037**	−0.057410	0.3851	0.515478	**<0.001**	0.524864	**<0.001**
- in [20;60) years	−0.11665	0.1897	−0.26003	**0.00303**	0.41222	**<0.001**	0.3593	**<0.001**
- in <20 or ≥60 years	0.3772	**<0.001**	0.01824	0.85489	0.56325	**<0.001**	0.6047	**<0.001**

The significant associations between features are marked in bold.

TABLE 6 | Characteristics of speed and accuracy performance in gender groups.

Variable	**Total** $n_1 = 231$		**Female** $n_2 = 134$ (58.01%)	**Male** $n_3 = 97$ (41.99%)	p_{2-3}
Age	40.96	[24.87–59.76]	47.3±20.08	33.73 ± 21.98	**<0.0007**
DMT	93.84	[63.6–122.43]	93.46 ± 58.5	95.18 ± 46.4	0.2835
TRVI	61.9	[34.7–96.0]	63.85 ± 58.98	59.1 ± 50.62	0.0767
AC	1.08	[1.01–1.19]	1.1 ± 0.19	1.04 ± 0.19	**<0.001**
SVMR_mean	245.52	[219.63–285.83]	249.55 ± 57.56	237.96 ± 61.31	**<0.0147**
CVMR_mean	350.75	[307.45–395.57]	356.04 ± 81.17	345.08 ± 77.4	**<0.0259**
AST_mean	343.4	[311.05–412.35]	357.9 ± 68.9	336.9 ± 59.13	**<0.0114**
IRT_mean	413.8	[368.15–471.85]	427.65 ± 77.99	401.8 ± 77.11	**<0.0021**
RMO_mean	7.6	[−18.5–31.35]	7.95 ± 91.16	7.6 ± 58.05	0.4155
SVMR_variance	57.7	[41.09–80.82]	57.18 ± 47.22	58.27 ± 48.48	0.3557
CVMR_variance	91.81	[70.7–118.64]	91.44 ± 79.9	96.42 ± 67.4	0.3801
AST_variance	72.0	[53.55–115.6]	76.3 ± 60.08	68.1 ± 46.35	*0.0533*
IRT_variance	103.5	[76.75–156.85]	107.7 ± 64.11	97.1 ± 67.76	0.4734
RMO_variance	140.3	[84.7–224.35]	161.3 ± 107.07	125.1 ± 95.13	**<0.0014**
SVMR_mistakes	0.0	[0.0–2.0]	0.0 ± 1.57	1.0 ± 2.91	0.1577
CVMR_mistakes	2.0	[1.0–4.0]	2.0 ± 2.49	3.0 ± 2.23	**<0.0003**
AST_mistakes	1.0	[0.0–2.0]	1.0 ± 2.77	0.0 ± 1.93	**<0.0467**
IRT_mistakes	3.0	[1.0–6.0]	3.0 ± 3.95	3.0 ± 3.44	0.1936
RMO_mistakes	22.0	[18.0–24.0]	22.5 ± 4.25	20.0 ± 4.83	**<0.001**
SVMR_IES	253.7	[224.94–304.73]	256.33 ± 72.12	249.57 ± 172.73	**<0.0451**
CVMR_IES	382.34	[336.52–448.65]	381.8 ± 110.89	383.04 ± 92.34	0.2482
AST_IES	357.1	[318.6–443.48]	368.6 ± 124.48	339.96 ± 89.24	**<0.0063**
IRT_IES	458.25	[398.08–591.65]	493.5 ± 191.49	433.14 ± 182.49	**<0.0036**
Adolescents	48 (20.78%)		19 (14.18%)*	29 (29.9%)*	**<0.0078**
Young adults	64 (27.71%)		36 (26.87%)	28 (28.87%)	
Midlife adults	64 (27.71%)		39 (29.1%)	25 (25.77%)	
Older adults	55 (23.81%)		40 (29.85%)*	15 (15.46%)*	

**If the proportion of males and females in an age group is significantly different compared to other groups, such the group is marked with an asterisk. The significant differences between cohorts are marked in bold.*

girls in the adolescent group (29.9 vs. 14.18%; $p < 0.05$) whilst the number of elderly women studied was significantly higher compared to men (29.85 vs. 15.46%).

To perform the comparison we approximated the parameters of the model with the polynomial function of the degree one. We built the model for the overall study sample (**Figure 6A**) and for the values that are within the 15–85 percentile range separately (**Figure 6B**). The sample size decreased from 231 to 161 and 143 points for CVMR time and error rate, respectively.

Information on statistically significant differences between the intercepts and the slopes of the gender-specific models is in **Table 7**. Though both genders didn't show significant differences between reaction time speed decay throughout the lifespan, females seem to spend more time on the task on average, while performing it more accurately compared to men. After removing the outliers, the rate of the errors in females and males is significantly different ($p = 0.00000866$). Interestingly, males keep a stable accuracy level during the lifespan while the task performance accuracy drops with aging in the opposite gender.

4.4. The Predictive Potential of PTs to Identify the Values of Inverse Efficiency Scores

We built up a prediction model for the check-up if IES provides a summary of the findings obtained while testing individuals. The performance metrics of the regression model such as mean absolute error (MAE), root mean squared error

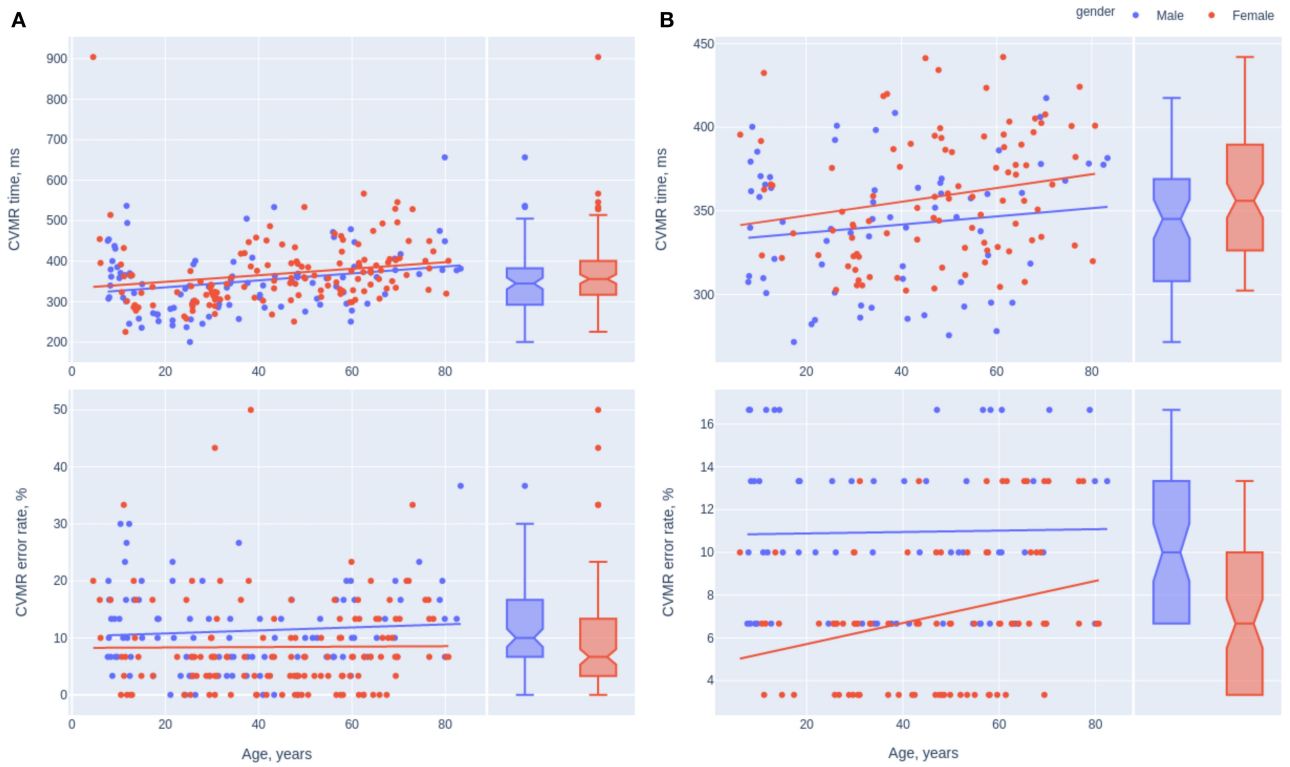

FIGURE 6 | Scatterplots of the mean reaction time and the error rate in CVMR test. The trendlines present the tendencies for different gender with **(A)** and without the outliers **(B)**.

TABLE 7 | The interaction coefficients for the comparison of the intercepts and the slopes of the models.

		A comparison of the intersepts		A comparison of the slopes	
		Estimate ± Std.Error	***p*-value**	**Estimate ± Std.Error**	***p*-value**
SVMR_IES vs. *CVMR_IES*		75.74 ± 23.12	**0.00113**	1.1423 ± 0.4979	**0.02224**
AST_IES vs. *IRT_IES*		82.6360 ± 29.8992	**0.005943**	1.1794 ± 0.6440	0.067686
SVMR_IES vs. *AST_IES*		33.5681 ± 23.5458	0.154650	1.9064 ± 0.5071	**0.000193**
CVMR_IES vs. *IRT_IES*		40.4584 ± 29.5649	0.17184	1.9434 ± 0.6368	**0.00241**
A cross-gender comparison					
CVMR_IES in females	Full dataset	−17.7913 ± 29.4919	0.5469	0.3271 ± 0.6461	0.61302
vs *CVMR_IES in males*	Without outliers	24.97785 ± 16.0458	0.122	−0.5732 ± 0.3475	0.101
CVMR_error_rate in females	Full dataset	0.602601 ± 0.692298	0.385	0.006794 ± 0.015167	0.655
vs *CVMR_error_rate in males*	Without outliers	6.09494 ± 1.31926	**0.00000866**	−0.04594 ± 0.02785	0.1013
CVMR_mean in females	Full dataset	−14.48187 ± 22.57078	0.5218	0.04636 ± 0.49450	0.9254
vs *CVMR_mean in males*	Without outliers	−6.9771 ± 14.0149	0.6193	−0.1674 ± 0.2971	0.5739

The significant differences between intercepts are marked in bold.

(RMSE), and coefficient of determination (R^2) are presented in **Table 8**. The graph in **Figure 7** reveals the prediction error for IES in POBA dataset whereas **Figure 8** presents the regression models' performance outcomes in different age groups.

4.4.1. Studying the Association of the Performance Speed, Accuracy, and Stability

The trendlines from scatter plots in **Figure 5** provide insight into the association of speed and accuracy of reaction. The examinees were set up neither for the time nor for the errorless

behavior. This means they conducted the test with their regular performance. On the one hand, this does not allow us to calculate the speed-accuracy trade-off out of the data. On the other hand, such a paradigm is more useful to look for any age-related changes in the performance characteristics.

We tried to estimate if IES reflects the overall status of an individual. The battery of tests used in POBA describes individual psychophysiological status. We built up a regression model of IES prediction out of the PS data and estimate its performance metrics. They are presented in **Table 8** and **Figure 7**.

5. DISCUSSION

5.1. The Age-Related Changes of Reaction Speed and Performance Accuracy

Conducting the cross-sectional study we tried to find out if the slowing of the RT goes uniformly along with the decline in accuracy while aging. First, we studied the age-related changes in the time estimates of the tests (e.g., reaction time and decision-making time). From Kruskal-Wallis test (see **Table 1**), it is evident that the reaction time and its variance was relatively high in adolescents, then it reduced in adults and raised up in the remaining age groups. The accuracy performance had the opposite tendency: it was low in the adolescents, then it improved in the young adults and dropped back in the following years of life. We assume that adolescents experience more difficulties with the concentration of attention, and this impacts the performance of the attention-related tasks, particularly *AST* and *IRT*. Additionally, we tested the median values of the asymmetry coefficient and the tests results that reflect information processing speed such as *DMT*, *TRVI*. Interestingly, TRVI was the only variable the age-related samples of which originated from a common distribution. In other words, there is no significant difference in the median values of TRVI of the age groups. Meanwhile, DMT had age-related significant differences in median values. Out of this, we decided to concentrate on studying IES score for the CVMR task rather than for IRT task the derivate of which (e.g., TRVI) vary insignificantly across age groups.

After studying the time estimates we shifted our study to the lifelong dynamics of the accuracy (e.g., ER). One may expect the same lifelong dynamics of the accuracy of CVMR performance as in the reaction time. However, it is different (**Figure 3**). The number of mistakes made performing CVMR test reduces till the age of 40 and only then it starts rising. Conceptually, there is no way to delay the age-related structural changes while aging but there is a way to compensate them by using the biological reserves. Moreover, the concept of reserve can be easily applied to brain aging. For example, the reaction time may slow with

TABLE 8 | Performance metrics on CVMR_IES prediction.

Regressor	MAE	RMSE	R^2	$\frac{MAE}{range(IES)}$, %
Gradient boosting	36.372	55.549	0.652	4.05
AdaBoost	37.102	56.313	0.662	4.13
K nearest neighbors	46.241	70.768	0.447	5.15
Lasso	30.310	45.635	0.768	3.37
Random forest	37.227	58.216	0.653	4.14
SVR non-linear	39.484	63.278	0.590	4.39

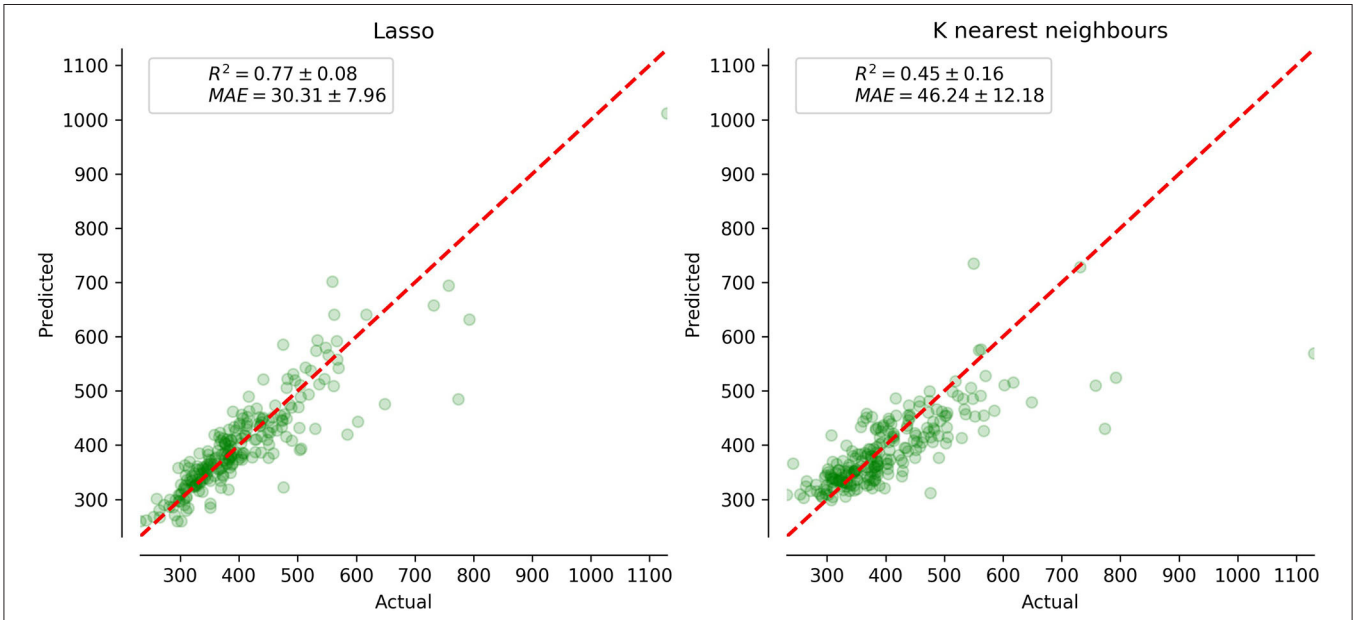

FIGURE 7 | The performance of the employed IES prediction regression models and visualization of prediction error using 10-fold cross-validation technique on POBA datasets.

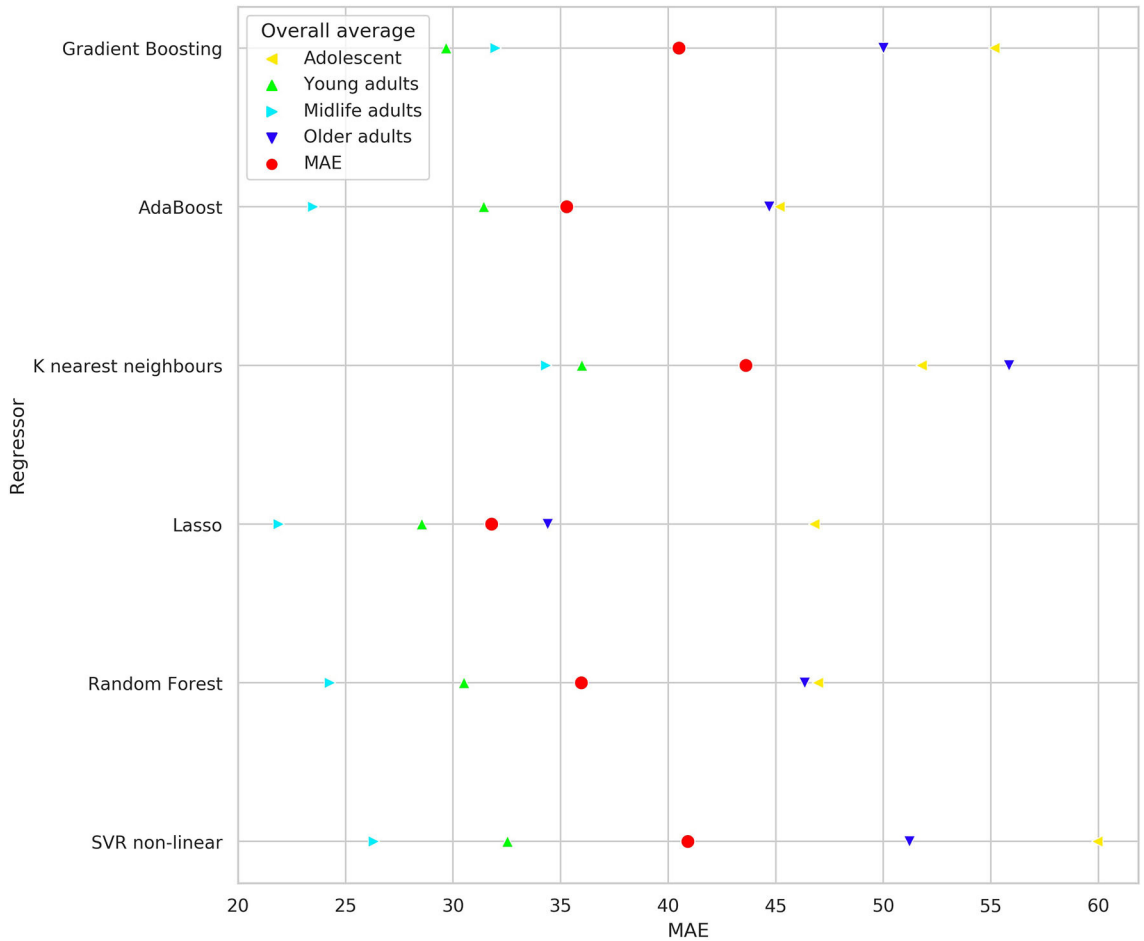

FIGURE 8 | Mean absolute error of employed regression models for predicting IES in both datasets. The colored triangles mark the data calculated for age groups, the red circles indicate the value for entire dataset.

age, while knowledge of world events may expand. According to Cullati et al. (2018), cognitive reserve is seen as actively acquired, and stimulating activities, like educational achievement over the life course, result in cognitive reserve maintaining or improvement. To test the hypothesis if the continuing education accounts for the decrease in the number of mistakes we may potentially use such proxies as the total number of years of education. However, they do not serve as the direct measure of intellectual functioning. It must be mentioned that participants at the age range of 40–50 years had the best test performance in terms of response accuracy. The error rate was <15% in almost all the cases. For this level of accuracy authors tend to analyze RT rather than ER, especially in case of the confusive findings when both RT and ER point in the same direction (Bruyer and Brysbaert, 2011). Furthermore, Akhtar and Enns (1989) suggest that IES better not used when ER >10%. There is no consensus on what is the threshold value of ER when researchers can rely on IES. Because of this, a study should not be limited to the analysis of the IES score. Moreover, the information on RT is not limited to its absolute value, the variance of RT across trials reflects the reaction stability. Such information is totally missing in IES.

5.2. The Association of the Proposed Efficiency Scores With the Age and Cognition

In total, the statements about the data encompassing the POBA dataset and the assumptions that come from their analysis is consistent with the concept of speed-accuracy trade-off and its age-related changes. Studying the lifelong tendencies of the tests (see **Figure 4**), we see that the IES scores for all the PT increase steadily with age except the SVMR_IES. Notably, the values of the reaction time for SVMR were scattered more than for other tests. This may result from the study methodology. We started testing people with the SVMR test as the easiest one. Although it is a simple one, some experience is required to get used to it. This assumption is in line with a formal model of decision making by Stone (1960) which supports the fact of the information accumulation over the course of perceptual decision-making (Stone, 1960; Heitz, 2014). It is common sense that adolescents if compared to other age groups have the lowest number of the total years of education. The lower education level and the attention deficit typical of this age

may result in the rate of adoption to the tasks we delivered. Because of this, the variance for reaction time for SVMR in adolescents was bigger than its mean value (SVMR_variance > SVMR_mean) unlike for the other tests (e.g., CVMR_variance < CVMR_mean, AST_variance < AST_mean, IRT_variance < IRT_mean). After removing the outliers, the negative trend for the lifelong dynamics of SVMR_IES was reversed. However, this did not change the age-related tendencies in the other tests (e.g., CVMR, AST, IRT). The significant reduction of the sample size makes it less representative and may hide tendencies that were remarkable in the full dataset. However, the removal of the outliers is required for the analysis of the lifelong changes in SVMR metrics. From **Table 2**, the age prediction out of the AST_IES and IRT_IES values has better performance if the model is trained with the full dataset rather than without them (e.g., $R^2 = 0.10976$ vs. 0.088361 for AST; 0.108747 vs. 0.06731 for IRT). To sum up, IES for PT undergo age-related changes that can be described as the neurocognitive slowing, i.e., the latencies increase steadily. We may consider IES as markers of the brain aging because they incorporate information both on RT and accuracy. We also showed the ambiguity of the removal of the dataset outliers for the reproducibility of the data.

As the battery of PT provides a set of time and accuracy estimates, several IES scores derive from diverse task paradigms. This rises up a question of the comparison of the scores and their association with the age. On **Figure 4B** it is well seen that the rate of age-related IES progression increased with the difficulty of the task (see the slopes values in **Table 2**). As seen from **Table 7**, there is a significant difference in the slope value in SVMR_IES compared to CVMR_IES ($p = 0.02224$). This is in consistency with the other researchers who found a difference in the time for processing the simple reaction task (0.5 ms/yr) vs. the complex one (1.6 ms/yr) (Fozard et al., 1994). At the same time, it is hard to explain why the slopes for AST and IRT do not differ significantly ($p = 0.067686$). The interfering objects in IRT test add the switching condition which is absent in AST similarly like CVMR has a switching condition while SVMR does not. Because of the switching condition we expected that the lifelong dynamics of IRT should differ significantly from AST. As it was not so, we decided to proceed with the exploration of the CVMR_IES, its age-related trends, and gender-specific features.

Another way of comparing the proposed IES for PT is by using the cognitive approach. We looked for the score that has the strongest informative power to reflect changes in cognitive subdomains. From **Table 3**, the association of the psychophysiological estimates of the information processing speed (DMT, TRVI) with CVMR_IES is more tight ($p \leq 0.001$) than with IRT_IES (p-value p to 0.03). This was another reason for us to decide to preserve CVMR_IES as the major marker of the speed-accuracy performance for the battery of tests.

Thus, it looks promising to propose IES for PT to cognitive studies in aging neuroscience for a set of reasons. First, there is an evident dynamics of the indices throughout the lifespan. Second, neurocognitive slowing estimated with IES-like indices correlates with task difficulty. Finally, the indices are reflective of cognitive subdomains (e.g. switching and inhibition).

5.3. The Relationship Between the Reaction Time and Accuracy

5.3.1. Age-Related Aspects

The concept of SAT implies the presence of negative correlation between RT and ER. However, many authors use IES without the preliminary checkout if there is a negative correlation between RT and ER. In our dataset, the "adolescents" and the "older adults" shared one tendency regarding the speed-accuracy ratio without SAT, while the other two groups shared the opposite trend (see **Figure 5A**). Both at the time of active developmental changes *in adolescence* and during ongoing atrophic changes *in elderly* there is the tendency toward the rise of the number of mistakes while slowing the reaction. The quicker the task is done the better the accuracy is or vice versa. We hypothesize that there are two options when we talk about an 'older adult: the person is either cognitively preserved and has a good task performance or the individual is somehow cognitively impaired, which reduces all metrics of performance (e.g., both the reaction time and the reaction accuracy). The examinees in the age range from 20 to 60 show the opposite relationship between the speed and accuracy. Slow reaction (long reaction time) is associated with the low number of mistakes which makes sense for the regular condition of an adult. So, in this age group we see the presence of SAT. In our battery of tests CVMR was the only one with the significant negative association between RT and ER in the subcohorts of young and midlife adults taken together (see **Table 5**). This goes in line with a study of Townsend and Ashby who advised to use IES only in case of a high and linear correlation between RT and ER (Townsend and Ashby, 1978; Townsend et al., 1983). However, an experiment by Ferrand evidenced that a positive correlation between RT and ER does not necessarily mean that more variance will be explained better in the IES rather than in RT measures (Ferrand et al., 2010; Bruyer and Brysbaert, 2011). This is exactly what we see in **Tables 3**, **5**, i.e., the variance of ER is explained better in the CVMR_IES ($r = 0.300759$; $p < 0.001$) than in CVMR_mean ($r = -0.057410$; $p = 0.38$). Many researchers do not follow the recommendations of Townsend and Ashby to check if RT and PE are positively correlated before using IES (Goffaux et al., 2005; Jacques and Rossion, 2007; Kuefner et al., 2010). Some authors do not analyze PE and ER but go only for the analysis of IES (Minnebusch et al., 2008). Other authors report SAT as an issue which rises up when the examinee is set up for either time or errorless performance (Murphy and Klein, 1998; Kennett et al., 2001). But there is no common solution accepted by the international research society.

5.3.2. Gender-Related Aspects

One may expect that psychological traits of men and women result in different age-related dynamics of RT or ER. The age-related changes of the task performance look similar in both gender disregarding the fact whether we analyze the total study cohort (**Figure 6A**) or the reduced sample after removing the outliers (**Figure 6B**). In general, the rate of both the neurocognitive slowing and the accuracy reduction is more pronounced in men rather than in women. In both samples with or without outliers we observed insignificant advantage in RT for

male participants. In part this reproduces the results of a study by Adam (1999) who also found a near-significant overall reaction time advantage for male participants. The authors suggested that gender difference in reaction time performance may reflect differences in processing strategy. Other researcher achieved confusing results that there was no significant difference in RT between people of both gender during a unimanual speed-accuracy task, however during a bimanual task, the reaction time of both hands was significantly longer in women than men (Mickevičienė et al., 2011). We didn't find significant differences in the slopes of the linear models describing reaction time and error rate (see **Table 7**). After removing the outliers, the average number of mistakes was significantly higher in males than in females. The results stay in line with the data from Larson et al. that females and subjects high on neuroticism made significantly fewer errors in a choice reaction-time task (Larson and Saccuzzo, 1986). In an experiment with the bimanual task the accuracy of the left hand was significantly greater in men than women (Mickevičienė et al., 2011). Thus, we can report the presence of some gender-dependent features of task performance. According to our data and some references (Dane and Erzurumluoglu, 2003; Barral and Debû, 2004), women perform more slowly and accurately than men in the speed-accuracy task.

5.4. The Informative Value of the Inverse Efficiency Score in PT

Hypothetically, IES may serve as a marker of psychophysiological status. If so, machine learning algorithms should be able to predict it out other PT results. **Table 8** illustrates the predictive potential of PTs to identify the values of inverse efficiency scores. The performance metrics are good: the proportion of MAE to the range of IES values is low for all regressors (3.37–5.15%). Lasso regressor has the best performance, the R^2 value for it is close to 1.0, and this justifies the high accuracy of the regression model. The graph in **Figure 7** visually evidences low prediction error for IES in POBA dataset. The regressors with the best (Lasso) and the worst (K nearest neighbors) are shown. Visually, the difference between them is insignificant, i.e., all the created regression models are quite reliable. **Figure 8** presents the prediction performance of the regression models; the performance varies regarding the age. MAE is maximal for adolescence and a bit lower for older adults group. We can explain this by the individual rate of cognitive changes at the beginning and at the end of life. This assumption looks convincing cause neurodevelopment and aging are highly individual processes. In POBA dataset, all the models provide the best prediction for young and middle-aged adults. This supports the presence of SAT in the age groups mentioned above and the applicability of IES to them.

Developing the IES for clinical use may improve the currently existing strategies for the early diagnostics of dementia. The supposed benefits are the following. First, the application of the tests with SAT will provide physicians and clinical psychologists quantitative metrics of the cognitive status. The quantification is crucial for follow-up studies. Second, deploying machine learning algorithms for predicting biological age may help to organize the population screening for dementia to find suspicious cases at an early stage. The tasks that include the SAT condition seem more informative because they may reflect cognitive impairment in a set of domains of executive functioning. To make the screening more proficient, we suppose to utilize the machine learning approach for fusing the evidence from such psychophysiological tests with the one that comes from radiological findings (e.g., brain MRI).

6. LIMITATION OF THE STUDY

The known limitation of the study lies in the speed-accuracy ratio and payoff conception itself. It turns out that the assumption on the relative time cost of correct decision works for the summary statistics of the overall results obtained throughout the entire test. But if analyzed separately, the average reaction time for the error responses is faster than that for the correct ones.

Moreover, the time cost of making an error depends on the level of accuracy at which one is operating at the moment. An error saves more time at high levels of accuracy than at low levels. This means that the initial intent to figure out the time cost of making an error does not make sense. It is rather meant to estimate how long it requires to improve the confidence of the confidence of the examinee's judgment by a factor of two or ten compared to the present level of performance.

7. CONCLUSION

- IES score is potentially a clinically useful metric for summarizing the overall efficiency of decision making. It can be a particularly reliable tool when applied to psychophysiological test results as it reflects speed-accuracy performance and age-related changes. As a dependent variable for the complex visual-motor reaction test, IES is the best out of four newly proposed indices. IES accounts for different cognitive subdomains and may reflect their disproportional changes throughout the lifespan. This encourages us to explore psychophysiological test results utilizing machine learning models that can be designed as a reliable computer-aided detector of cognitive changes at an early stage.
- The examinees under 20 and over 60 years of age share one tendency regarding the speed-accuracy ratio without speed-accuracy trade-off. Both at the time of active developmental changes in adolescence and during ongoing atrophic changes in elderly there is a tendency toward a rise of the number of mistakes while slowing the reaction. In the age range from 20 to 60 the relationship between the speed and accuracy is opposite. In our battery, complex visual-motor reaction is the only test with the significant negative association between reaction time and error rate in the subcohort of young and midlife adults taken together.
- On average, women perform more slowly and accurately than men in the speed-accuracy task, however most of the gender-related differences are insignificant. Interestingly, males keep a stable accuracy level during the lifespan while the task performance accuracy drops with aging in the opposite gender.
- The IES index for PT is a reliable index that describes the individual psychophysiological status. Using results of other

psychophysiological tests, we predicted IES values for the visual-motor reaction with high accuracy ($R^2 = 0.77 \pm 0.08$; mean absolute error/IES range = 3.37%). The regression model shows the best performance in the cognitively preserved population groups of young and middle-aged adults (20–60 yrs). The individual rate of neurodevelopment in youth and atrophy in elderly results in worse prediction performance of the regression model.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The POBA dataset generated for this study can be found at the site of Data Analytics Group at: https://bi-dac.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by United Arab Emirates University Human Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YS and TH contributed to the conceptual idea of the paper. YS formulated the objectives and wrote the manuscript. TH performed the statistical analysis, prepared the figures and tables for data presentation and illustration. KG with NZ and TA contributed to the literature review and data analysis. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Neural Correlates of Age-Related Changes in Precise Grip Force Regulation: A Combined EEG-fNIRS Study

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Motor control is associated with suppression of oscillatory activity in alpha (8–12 Hz) and beta (12–30 Hz) ranges and elevation of oxygenated hemoglobin levels in motor-cortical areas. Aging leads to changes in oscillatory and hemodynamic brain activity and impairments in motor control. However, the relationship between age-related changes in motor control and brain activity is not yet fully understood. Therefore, this study aimed to investigate age-related and task-complexity-related changes in grip force control and the underlying oscillatory and hemodynamic activity. Sixteen younger [age (mean ± SD) = 25.4 ± 1.9, 20–30 years] and 16 older (age = 56.7 ± 4.7, 50–70 years) healthy men were asked to use a power grip to perform six trials each of easy and complex force tracking tasks (FTTs) with their right dominant hand in a randomized within-subject design. Grip force control was assessed using a sensor-based device. Brain activity in premotor and primary motor areas of both hemispheres was assessed by electroencephalography (EEG) and functional near-infrared spectroscopy (fNIRS). Older adults showed significantly higher inaccuracies and higher hemodynamic activity in both FTTs than did young adults. Correlations between grip force control owing to task complexity and beta activity were different in the contralateral premotor cortex (PMC) between younger and older adults. Collectively, these findings suggest that aging leads to impairment of grip force control and an increase in hemodynamic activity independent of task complexity. EEG beta oscillations may represent a task-specific neurophysiological marker for age-related decline in complex grip force control and its underlying compensation strategies. Further EEG-fNIRS studies are necessary to determine neurophysiological markers of dysfunctions underlying age-related motor disabilities for the improvement of individual diagnosis and therapeutic approaches.

Keywords: electroencephalography, functional near-infrared spectroscopy, aging, motor control, motor recovery, neuroplasticity

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INTRODUCTION

The ability to adapt our actions quickly to changes in the environment is a key function of human motor skills (Hermsdörfer et al., 2003). One of the most sophisticated characteristics of fine motor skills is the precise grip force regulation according to the physical requirements of a manipulated object, which is necessary for the successful performance of various everyday activities (Hermsdörfer et al., 2003; Voelcker-Rehage and Alberts, 2005; Parry et al., 2019). When gripping an object, radial forces are exerted that are large enough to prevent the object from slipping while ensuring that it does not break. Tangential forces are applied to move the object along the desired path (Flanagan and Wing, 1995; Haggard and Wing, 1995). Thus, regulation of precise grip force requires the coupling of radial and tangential grip forces (Hermsdörfer et al., 2000; Nowak et al., 2001; Rand et al., 2004), which is based on visuomotor transformation processes and the intact integration of sensory feedback within the motor-cortical network (Hermsdörfer et al., 2003; Prodoehl et al., 2009). Functional activity in motor-cortical areas can be quantified as cerebral hemodynamics using brain imaging techniques such as functional magnetic resonance imaging (fMRI) and functional near-infrared spectroscopy (fNIRS; Agbangla et al., 2017) or as electrical signals using the electroencephalography (EEG; Hamacher et al., 2015). It is suggested that synchronous oscillatory activity in the alpha (8–12 Hz) and beta (12–30 Hz) ranges within the motor-cortical network represents the basic mechanism for functional communication underlying the motor control process (Davis et al., 2012; Wach et al., 2013; Pollok et al., 2015). The alpha rhythm reflects cortical processes during the awake and attentive-resting states, and the amplitude of the signal is suppressed by sensory stimulation or during the motor control process (Engel and Fries, 2010). Beta oscillations are attenuated during voluntary movements, which is known as movement-related beta desynchronization (MRBD; Zaepffel et al., 2013). After the completion of a movement, beta oscillations are more pronounced, which has been interpreted as increased inhibition to maintain the status quo of the motor system (Pfurtscheller et al., 2006; Koelewijn et al., 2008; Engel and Fries, 2010). Previous studies have shown that oscillatory activity and cerebral hemodynamics are inversely related during the motor control process, that is, hemodynamic activity increases with a decrease in alpha and beta oscillations (Lachert et al., 2017). Changes in brain activity during the motor control process have been observed in various cerebral structures (Zaepffel et al., 2013), mainly in sensorimotor areas with a contralateral predominance (Taniguchi et al., 2000). Concerning grip force tasks, as an example, young healthy people exhibited increased hemodynamic activity in the sensorimotor cortex (SMC) and in the premotor cortex (PMC; Ehrsson et al., 2000; Cramer et al., 2002; Wriessnegger et al., 2017). The SMC contralateral to the active hand showed the highest hemodynamic activity (Wriessnegger et al., 2017) confirming the involvement of the SMC during movement execution (Leff et al., 2011). Furthermore, the prefrontal cortex (PFC), supplementary motor area (SMA), and PMC bilaterally as well as the cerebellum were found to be involved in grip force execution (Dai et al., 2001). Thereby, the SMA represents a key structure controlling the motor-cortical network while driving the regulation of grip forces by promoting and suppressing its activity (Grefkes et al., 2008a). Alterations in brain functions within the motor-cortical network can be caused by neurological diseases (Schnitzler and Gross, 2005; Grefkes et al., 2008b; Engel and Fries, 2010; Kiyama et al., 2014). According to previous studies, reduced inter-and intra-hemispheric connectivity between the SMA and the primary motor cortex (Grefkes et al., 2008b), upregulation of the parieto-frontal network activity in the stroke-lesioned hemisphere (Bönstrup et al., 2018), and pathological increases in beta oscillations (Rossiter et al., 2014a) were correlated significantly with motor deficits (Grefkes et al., 2008b; Rossiter et al., 2014a; Bönstrup et al., 2018).

More than one billion people worldwide suffer from neurological diseases. The number will increase in the coming years because of the aging population (Semprini et al., 2018). Early signs of age-related diseases are altered brain structures and synaptic functions ranging from pathological oscillations and altered functional connectivity to reduced gray matter volume (Minati et al., 2007; Ishii et al., 2017; Xifra-Porxas et al., 2019). During grip force control, older adults exhibited higher beta power, greater (more negative) MRBD (Xifra-Porxas et al., 2019), and significantly higher hemodynamic activity in subcortical areas and PMC than did young healthy adults (Noble et al., 2011). Deficits in fine motor skills and behavioral declines are most often the consequences of these changes (Voelcker-Rehage and Alberts, 2005; Grefkes et al., 2008b; Engel and Fries, 2010; Kiyama et al., 2014). However, the nature of the relationship between age-related changes in brain activity patterns and declines in grip force regulation has not been fully elucidated. Previous studies concerning age-related deteriorations in motor functions used tasks such as finger tapping (Naccarato et al., 2006), key pressing (Mattay et al., 2002), index finger abductions/adductions, or wrist extensions/flexions (Hutchinson et al., 2002), thus focusing on repetitive movements that are limited in elderly individuals, when activities of everyday lives that require the regulation of grip forces must be performed (Diermayr et al., 2011; Bock and Steinberg, 2012). It is, therefore, important to investigate how the precise regulation of grip forces is related to brain activity and is influenced by the aging process.

In this study, EEG and fNIRS, which are well-established, promising electro/neurophysiological techniques that are affordable, easy to implement, and easy to integrate into the diagnosis of neurological conditions in patients of all age groups, were used (Makeig et al., 2009; Herold et al., 2017). Knowledge of how grip force regulation and hemodynamic/oscillatory brain activity are related and altered according to age provides crucial information for individualization of therapy protocols or advancement of interventions such as neurofeedback, brain-machine interfaces, or noninvasive brain stimulation (NIBS; Gassert and Dietz, 2018; Semprini et al., 2018; Berger et al., 2019a). More specifically, for example, identifying pathological hypo-/hyperactivity or changes in oscillatory activity while applying fine motor skills could guide future NIBS or training protocols to enable the administration of target-oriented and

individualized interventions (Teo et al., 2016; Berger et al., 2018; Gassert and Dietz, 2018).

Therefore, the central purpose of this study was to investigate age- and task-related changes in hemodynamic and oscillatory activity during two visually guided force tracking tasks (FTTs) of different complexities. The easy FTT requires static grip force, whereas the complex FTT requires grip force-related characteristics necessary in everyday life settings (e.g., fine-tuning and quick adjustments of grip forces according to changing demands, such as when grasping objects to prevent slipping). As discussed above and based on the findings of previous studies that focused on task complexity (Holper et al., 2009; Wriessnegger et al., 2017) or age-related changes (Voelcker-Rehage and Alberts, 2005; Noble et al., 2011; Rossiter et al., 2014a,b; Xifra-Porxas et al., 2019), we hypothesized differences between the behavioral and neuronal factors associated with the two FTTs and between the younger and older adults (Noble et al., 2011) as follows: we expected: (1) that the complex FTT, which requires the regulation of grip force, results in higher inaccuracies in grip force control and greater brain activity in motor-cortical areas than does the easy FTT; and (2) that the older adults show greater deficits in precise grip force control and changes in sensorimotor activity during complex FTT than did younger adults.

MATERIALS AND METHODS

This study was performed following the ethical standards laid down in the Declaration of Helsinki. Experimental procedures were performed according to the recommendations of the Deutsche Gesellschaft für Psychologie (DGP) and approved by the local ethics committee of the Johannes Gutenberg-Universität Mainz. All participants were informed about the study-related contents before obtaining written informed consent before the initiation of the experiment.

Participants

Sixteen younger male participants aged 20–30 years (mean age: 25.44 ± 1.93 years) and sixteen older male participants aged 50–70 years (mean age: 56.75 ± 4.71 years) without any hand pathologies and extremity injuries were recruited to participate in this study. All participants were right-handed according to the Edinburg Handedness-scale (Oldfield, 1971), had no neurological or psychological disorders, and had a normal or corrected-to-normal vision. They were asked to disclose information on preexisting neurological, psychological, and medical conditions and on drug and alcohol/caffeine intake during the previous week.

Experimental Equipment and Data Acquisition

Grip Force Measures

The ability of precise grip force control was measured using the FTT, which is a well-known diagnostic tool used to investigate specific aspects of force control, that is, force generation and force release (Voelcker-Rehage and Alberts, 2005). It consists of a monitor and a force transducer for the dominant right hand (LIME medical GmbH, Germany). Four rails for the index, middle, ring, and little fingers guarantee a standardized position of the hand (see **Figure 1A**). The participants were instructed to use the power grip to match a curve representing the applied force of their dominant (right) hand to a predefined target route as accurately as possible. The participants and the target route were displayed on a screen at approximately 80 cm. The participants aimed to make as few deviations as possible. A deviation is defined as the absolute difference between the applied force and the optimal target force. All forces (the participant's applied grip force; predefined, optimal target force; and the resulting deviation) were measured in Newton (N) and calculated with a sampling rate of 10 Hz.

EEG Measures

EEG was recorded using Brain Vision Recorder 1.2 Brain Products, Germany equipped with 32 active Ag/AgCl electrodes, at a sampling rate of 1,000 Hz (notch filter at 50 Hz). Head dimensions were measured individually, and caps were adapted to the corresponding head sizes. Data were recorded using 28 electrodes placed on the scalp at Fp1, Fp2, F7, F3, Fz, F4, F8, FC5, FC1, FC2, FC6, T7, C3, Cz, C4, T8, CP5, CP1, CP2, CP6, P7, P3, Pz, P4, P8, O1, Oz, and O2 according to the international 5:10 system (Jurcak et al., 2007; see **Figure 1B**). Vertical and horizontal electrooculograms were recorded with four additional electrodes placed next to the left and right eye and above/below the right eye to detect eye movements and eyelid artifacts. The reference and ground electrodes were placed at the nose tip and AFz location, respectively. Impedance was maintained below 5 kΩ by using a SuperViscTM electrode filled with gel (EASYCAP GmbH, Germany) for conductivity.

fNIRS Measures

For fNIRS measurement, cerebral oxygenation changes were recorded using a near-infrared optical tomographic imaging device (NIRSport, NIRx, Germany, Wavelengths: 760 nm, 850 nm, sampling rate: 7.81 Hz). The methodology and underlying physiology are explained in detail elsewhere (Obrig and Villringer, 2003). A total of 16 optodes (eight emitters, eight detectors) were placed at 3 cm intervals above the motor cortex according to the international 5:10 system (Jurcak et al., 2007), resulting in 23 channels (source-detector pairs; see **Figure 1B**). Channel positions covered identical regions of both hemispheres, including the SMC [Brodmann Area (BA) 1–4] and SMA/PMC (BA6).

Experimental Design and Procedure

To control the maximum voluntary contraction (MVC) of the participants, a standardized Grip-Strength-Test was performed before the experiment. Participants sat upright in a standardized position on a height-adjustable chair (Massy-Westropp et al., 2011). The right upper arm was abducted, the elbow joint was flexed at 90°, the wrist was in a neutral zero position and the arm was held close to the body but not resting on the armrest of the chair. Using an electronic hand dynamometer (TL-LSC100, Trailite), three values of MVC were measured with the dominant right hand. The rest period in between was 1 min.

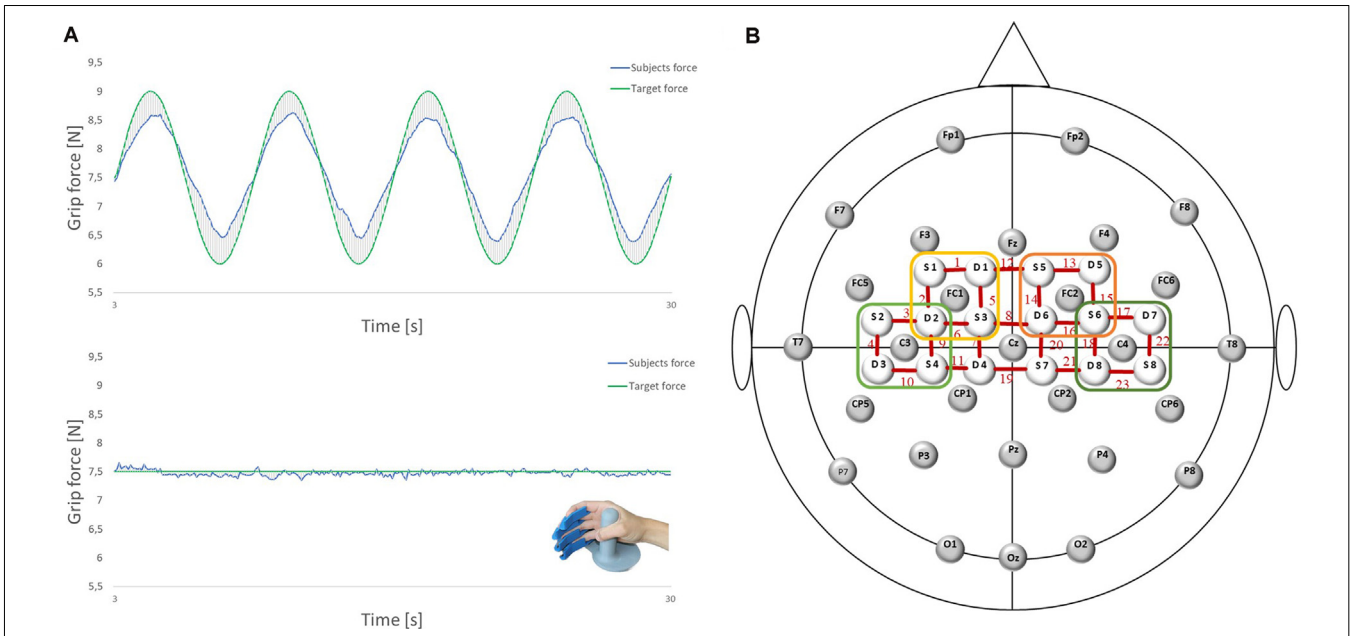

FIGURE 1 | (A) Force tracking tasks (FTTs) and the force transducer for the right dominant hand (LIME medical GmbH, Germany): complex FTT (top); easy FTT (bottom); green: target force; blue: subjects force. **(B)** Electroencephalography (EEG)/functional near-infrared spectroscopy (fNIRS) Montage: 28 EEG electrodes and 16 fNIRS optodes placed according to the 5:10 EEG system. Regions of interest are marked. Left premotor cortex (PMC): FC1 and Channels 1, 2, 5, 6 (yellow); right PMC: FC2 and Channels 13, 14, 15, 16 (orange); left sensorimotor cortex (SMC): C3 and Channels 3, 4, 9, 10 (light green); right SMC: C4 and Channels 17, 18, 2, 23 (dark green).

Participants performed two visually-guided grip-FTTs in a randomized order: in one task (easy FTT), participants had to follow a target force depicted as a straight line at 7.5 N by maintaining a constant grip force with the right dominant hand. In the other task (complex FTT), the target force changed over time in the form of a sinusoidal curve averaged to 7.5 N (6–9 N). Participants had to regulate and adapt their grip forces (N) continuously to the target value (see **Figure 1A**). Each FTT consisted of six task trials, with each trial lasting 30 s and alternating with six trials of rest (30 s; see **Figure 2**). During the trials of rest, participants were instructed to watch a fixation cross on the screen. Before and after each FTT, brain activity was recorded at rest for 30 s each with eyes closed and open. During the measurements, participants were instructed to avoid head movements and talking to reduce motion and physiological artifacts (Vitorio et al., 2017). For familiarization, two trials of the easy FTT and two trials of the complex FTT were performed before the experiment.

Data Processing

Grip Force Measures

For the MVC, the mean of the three values was collected from each participant. Then, the arithmetic mean was calculated for the younger and older adults. Regarding the FTT, the participant's applied grip force, the predefined, optimal target force, and their absolute deviations were measured for each FTT trial. Data from the first 3 s of each trial were excluded from further analysis considering the initial force adaptation at the onset. Then, the arithmetic mean of the absolute deviation values was computed, which represents the inaccuracy in grip force control (Gölz et al., 2018). Normal distribution was examined using the Shapiro–Wilk test ($p \geq 0.05$). A two-way repeated-measures analysis of variance (rmANOVA) using the factors TASK (easy FTT, complex FTT) and GROUP (younger adults, older adults) was conducted to evaluate changes in grip force control owing to task complexity and aging. Bonferroni-adjusted *post-hoc* analyses were used to identify significant differences.

EEG Measures

Electrophysiological data were preprocessed using the Brain Vision Analyzer 2.0 (Brain Products, Gilching, Germany) by applying a 0.5 Hz high-pass filter and a 50 Hz low-pass filter. Horizontal and vertical eye movements were corrected using the Gratton and Coles methods (Gratton et al., 1983). Any remaining artifacts were removed using a semiautomatic inspection tool by setting the maximum allowed gradient voltage step at 50 mV/ms and the maximally allowed amplitude at 100 mV. Further artifacts were visually identified and manually rejected so that only segments containing no artifacts were included for further processing. For the evaluation of changes in oscillatory activity over time, the two task conditions (easy FTT and complex FTT) and the rest condition were subdivided into six trials of 30 s. Each condition was segmented into 1 s epochs, a fast Fourier transformation [Maximum Resolution (0.977 Hz); Power (μV^2); No window] was computed, and frequency spectra of the artifact-free segments were averaged for each condition. Then, oscillatory mean activities (mV) were calculated for the alpha

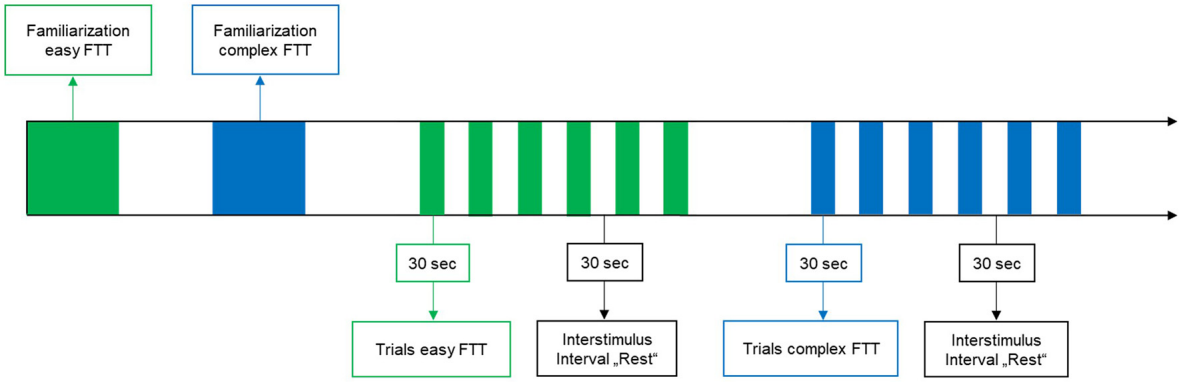

FIGURE 2 | Study design.

(8–12 Hz) and beta (13–30 Hz) ranges. Since suppressed beta activity relative to rest is associated with movement execution (Xifra-Porxas et al., 2019), the MRBD was calculated in addition to the absolute power. Therefore, beta activity at rest as well as the difference of beta activity between each FTT and rest was calculated and statistically analyzed. Following the fNIRS channels and the corresponding regions of interest (ROI), the following EEG electrodes were used for further statistical analysis: left SMA/PMC, FC1; right SMA/PMC, FC2; left SMC, C3; right SMC: C4 (see **Figure 1B**).

fNIRS Measures

Raw brain oxygenation data were preprocessed and analyzed using the time series analysis routine within the MATLAB-based NIRSlab analysis package (v2017.05, Nirx Medical Technologies, Glen Head, NY, USA; Xu et al., 2014) according to the current recommendations (Herold et al., 2017; Vitorio et al., 2017). Concentration changes of oxygenated hemoglobin (Hboxy) and deoxygenated hemoglobin (Hbdeoxy) were visually inspected concerning transient spikes and abrupt discontinuities representing the two most common forms of movement artifacts in fNIRS data. To achieve smoothening, epochs that contain discontinuities (or "jumps") or long-term drifts were corrected, and spikes were replaced by the nearest signal (Xu et al., 2014). Then, a band-pass filter (0.01–0.2 Hz) was applied to remove slow drifts and high frequencies related to breathing, respiratory or cardiac rhythm, vasomotion, or other movement artifacts (Koenraadt et al., 2014). After preprocessing, parameters such as wavelengths (WL1 = 760 nm; WL2 = 850 nm), differential pathlength factors (7.25 for WL1; 6.38 for WL2), interoptode distances (3 cm), and background tissue values (totHb: 75 μM; MVO2Sat: 70%) were specified, and the time series of Hboxy/Hbdeoxy concentration changes (ΔHboxy/ΔHboxy) were computed using the modified Beer-Lambert law (Cope et al., 1988; Sassaroli and Fantini, 2004). Afterward, ΔHboxy data of the easy and complex FTT trials (30 s) were exported to analyze their hemodynamic responses. Averaged baseline concentration values of 15 s rest before each trial were subtracted from the task-evoked concentration measurements to account for time-dependent changes in cerebral oxygenation (Vitorio et al., 2017). ΔHboxy was calculated for ROI (left SMA/PMC: Channels 1, 2, 5, 6; right SMA/PMC: Channels 13, 14, 15, and 16; left SMC: Channels 3, 4, 9, and 10; right SMC: Channels: 17, 18, 22, 23) during the FTTs and was used as a marker for regional cortical activation because it is more sensitive to motor-related activities than is Hbdeoxy (Suzuki et al., 2004) and because it is an accurate indicator of hemodynamic activity (Strangman et al., 2002).

Statistical Analysis

The statistical analysis of data corresponding to oscillatory and hemodynamic activity was conducted using SPSS 23 (IBM, Armonk, NY, USA). The normality of distribution was examined using the Shapiro–Wilk test ($p \geq 0.05$). Oscillatory mean activities and averaged ΔHboxy values corresponding to both the FTTs were computed for each subject and ROI (Herold et al., 2017; Vitorio et al., 2017). A three-way rmANOVA with the factors TASK (easy FTT, complex FTT), ROI [ROI1: left SMA/PMC (BA6); ROI2: right SMA/PMC (BA6); ROI3: left SMC (BA1–4); ROI4: right SMC (BA1–3)], and GROUP (younger adults, older adults) was conducted to evaluate changes in oscillatory and hemodynamic activity owing to task complexity, brain regions, and between the groups. In case of significant effects, Bonferroni-adjusted *post-hoc* analyses were used to identify significant differences between the tasks, ROI, and groups.

The overall level of significance was set to $p \leq 0.05$. Significant tendencies were defined as $p \leq 0.10$. Mauchly's test was used to check for any violations of sphericity. If a violation of sphericity was detected ($p \leq 0.05$) and a Greenhouse–Geisser $\varepsilon > 0.75$ existed, Huynh-Feldt corrected p-values were reported. Otherwise ($\varepsilon \leq 0.75$), a Greenhouse-Geisser correction was applied. Effect sizes were reported in the partial eta-square (η_p^2) for the significant main effects and interactions. According to Cohen (1988), we classified the effect sizes as follows: >0.01 for small, >0.06 for medium, and >0.14 for large effect sizes (Cohen, 1988).

To calculate the relationship between precise grip force control, oscillatory and hemodynamic activity, correlation analysis was performed for each task, region, and group. First, correlations were calculated for each task separately (e.g., correlation of inaccuracy and beta activity during the easy FTT). Second, differences between the easy FTT and the complex FTT were calculated. Then, difference values (Δ = complex FTT − easy FTT) were correlated (e.g., correlation of Δinaccuracy and $\Delta\beta$ activity) to analyze task complexity-related effects. Degrees of correlation between grip force control, oscillatory and hemodynamic activity were explored using the Pearson correlation coefficient: low, $r > 0.1$–0.3; medium, $r > 0.3$–0.5; strong, $r > 0.5$–0.7; very strong, $r > 0.7$–0.9; or perfect, $r > 0.9$. Furthermore, the correlations of younger and older adults were statistically compared (Eid et al., 2015).

RESULTS

Grip Force Measures

Regarding the MVC, no differences between the two groups were presented (younger adults: 45.45 ± 4.43, younger adults: 45.59 ± 7.67, $p = 0.951$). For the FTT, the two-way repeated-measures ANOVA (rmANOVA) using the factors TASK (easy FTT, complex FTT) and GROUP (younger adults, older adults) showed a statistically significant main effect of TASK ($F_{(1,30)} = 184.10$, $p < 0.001$, $\eta_p^2 = 0.86$) that indicates the incidence of a significantly higher inaccuracy during the complex FTT than during the easy FTT ($p < 0.001$). Furthermore, a significant main effect of GROUP ($F_{(1,30)} = 6.51$, $p = 0.02$, $\eta_p^2 = 0.18$) emerged that presented higher inaccuracies in grip force regulation in older adults than in young adults ($p = 0.02$). No significant interaction of TASK × GROUP was observed ($F_{(1,30)} = 2.24$, $p = 0.15$, $\eta_p^2 = 0.07$; see **Figure 3**).

fNIRS Measures

Statistical analysis of data on relative Hboxy concentration changes revealed a significant main effect of GROUP ($F_{(1,30)} = 4.36$, $p = 0.04$, $\eta_p^2 = 0.13$), indicating a significantly higher hemodynamic activity in the older adults than in the young adults. The significant main effect of ROI ($F_{(3,90)} = 3.56$, $p = 0.02$, $\eta_p^2 = 0.11$) showed significant differences between the cortical areas. Bonferroni-corrected *post-hoc* tests revealed significantly higher hemodynamic activity in the ipsilateral SMC than in the ipsilateral PMC ($p = 0.04$) and contralateral SMC ($p = 0.01$; see **Figure 4**). Otherwise, no further main effects or interactions were observed (all $p > 0.13$).

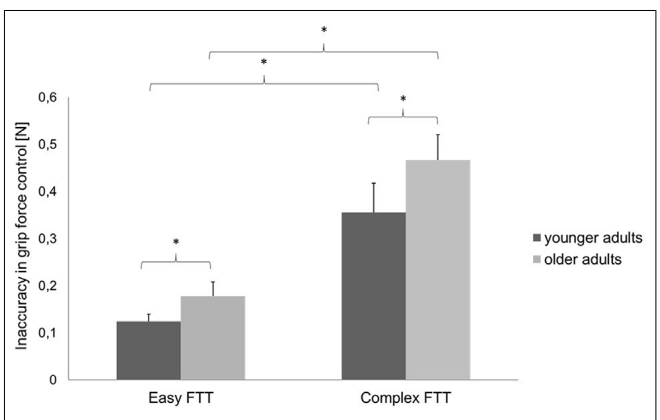

FIGURE 3 | Precision in grip force control (mean ± SD) of the younger and older adults during the easy and complex FTTs; *$p < 0.05$.

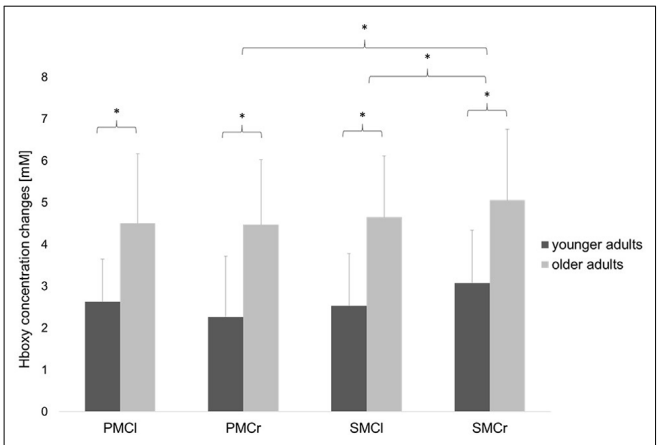

FIGURE 4 | Relative concentration changes of Hboxy (mean ± SD) in the four regions of interest averaged over the easy FTT and the complex FTT for the younger and older adults. *$p < 0.05$.

EEG Measures

Statistical analysis of data on oscillatory alpha activity (8–12 Hz) using a three-way rmANOVA with the factors TASK, ROI, and GROUP revealed neither main effects nor interaction effects (all $p > 0.10$). Concerning beta activity (13–30 Hz), the three-way rmANOVA showed a significant main effect of TASK ($F_{(1,30)} = 11.23$, $p < 0.001$, $\eta_p^2 = 0.27$), indicating a significantly higher beta activity during the complex FTT than during the easy FTT. The significant main effect ROI ($F_{(3,90)} = 5.65$, $p = 0.02$, $\eta_p^2 = 0.16$) revealed significantly higher beta activity in the left SMC than in the PMC and bilateral SMC. Furthermore, a statistically significant trend for the interaction of TASK × GROUP ($F_{(1,30)} = 3.10$, $p = 0.08$, $\eta_p^2 = 0.09$) emerged. Bonferroni-corrected *post-hoc* tests indicated significantly higher beta activity during the complex FTT than during the easy FTT in the group of older adults ($p = 0.001$; see **Figure 5**). Furthermore, analysis of data on beta activity during rest, the two-way rmANOVA with the factors ROI, and GROUP revealed neither main effects nor interaction effects (all $p > 0.10$). Regarding the MRBD, the three-way rmANOVA showed a significant main effect of TASK ($F_{(1,30)} = 11.04$, $p < 0.002$, $\eta_p^2 = 0.27$), indicating a significantly greater (more negative) MRBD during the easy FTT than during the complex FTT. The significant main effect ROI ($F_{(3,90)} = 5.41$, $p = 0.01$, $\eta_p^2 = 0.15$) revealed significantly higher beta suppression in the left SMC than in the left PMC. Furthermore, a statistically significant trend for the interaction of TASK × GROUP ($F_{(1,30)} = 3.11$, $p = 0.08$, $\eta_p^2 = 0.09$) and TASK × ROI × GROUP ($F_{(3,90)} = 2.78$, $p = 0.08$, $\eta_p^2 = 0.09$) emerged. Bonferroni-corrected *post-hoc* tests

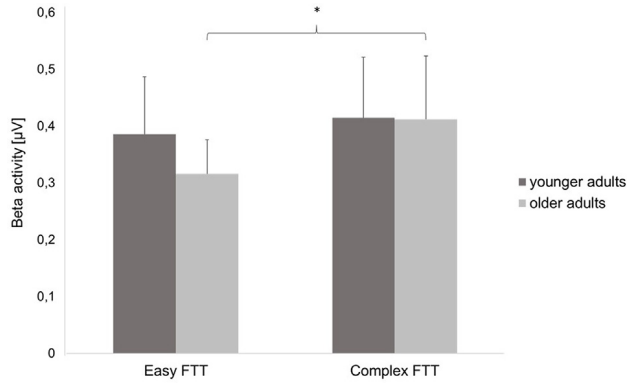

FIGURE 5 | Beta activity (mean ± SD) of the younger and older adults during the easy and complex FTT, $^*p < 0.05$.

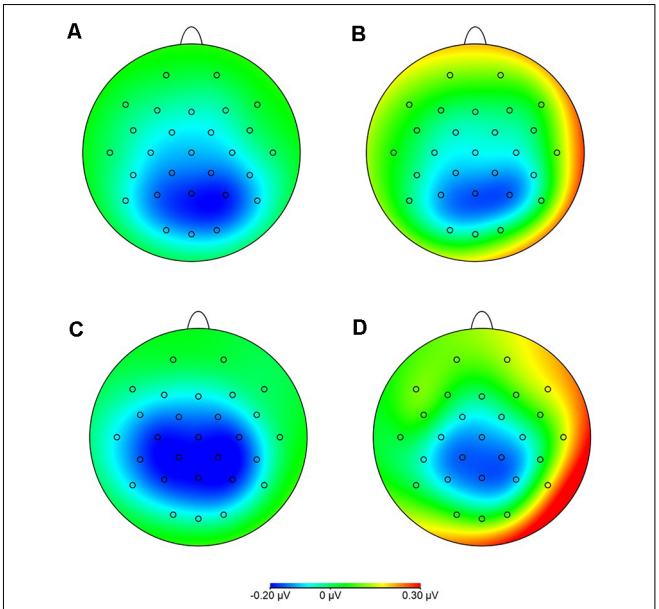

FIGURE 6 | Movement-related beta desynchronization (MRBD) **(A)** younger adults, easy FTT **(B)** younger adults, complex FTT **(C)** older adults, easy FTT **(D)** older adults, complex FTT.

indicated that older adults exhibited significantly greater MRBD on the SMC bilaterally during the easy FTT compared to the younger adults (left SMC $p = 0.04$, right SMC $p = 0.03$; see **Figure 6**).

Associations Between Precise Grip Force Control, Oscillatory and Hemodynamic Activity

For the separate analysis of the tasks, correlations between precise grip force control and oscillatory activity as well as precise grip force control and hemodynamic activity were statistically not significant. Regarding the relation of oscillatory beta activity and hemodynamic activity, three significant tendencies ($p < 0.08$) and one significant correlation of beta activity in the right SMC and hemodynamic activity in the left SMC emerged during the easy FTT for the younger adults ($r = 0.55$, $p = 0.03$). For the older adults, correlation analysis showed nine significant tendencies ($p < 0.08$) and one significant correlation of beta activity in the right PMC and hemodynamic activity in the right SMC during the easy FTT ($r = 0.50$, $p = 0.05$; see **Table 1**). During the complex FTT, no significant tendencies or correlations were presented. Concerning complexity-based differences (Δ = complex FTT − easy FTT), older adults showed a positive association with a trend between inaccuracy in precise grip force regulation and oscillatory beta activity in the left PMC ($r = 0.46$, $p = 0.07$), whereas young adults showed a non-significant negative association ($r = -0.32$, $p = 0.22$; see **Figure 7**). However, these correlations differed significantly between younger and older adults ($p = 0.03$). Thus, complexity-related deteriorations are accompanied by different motor cognitive processes in young and older adults.

DISCUSSION

This study aimed to investigate age-related changes in grip force regulation and associated changes in oscillatory and hemodynamic brain activity. The following is the summary of our findings: (1) age-related deteriorations in grip force control and increases in hemodynamic activity in motor-cortical areas occurred independently of task complexity and (2) deteriorations in grip force control owing to the task complexity was positively associated with left PMC beta activity in older adults and negatively associated in younger adults.

First, the older adults showed significantly greater inaccuracies in grip force control and significantly higher hemodynamic brain activity in all motor-cortical areas during the easy and complex FTTs than did the younger adults. These results confirm the existence of expected age-related deteriorations in grip force control (Voelcker-Rehage and Alberts, 2005) and increases in hemodynamic activity (Mattay et al., 2002; Naccarato et al., 2006; Kim et al., 2010; Noble et al., 2011). On one hand, aging is associated with diminished tactile sensations that reduce hand sensibilities, which lead to declines in fine motor skills (Cole, 1991; Bennett and Castiello, 1994; Voelcker-Rehage and Alberts, 2005). On the other hand, physiological brain aging is characterized by a loss of synaptic contacts and neuronal cell death that provokes age-dependent declines of sensory processing and motor control (Rossini et al., 2007; Boisgontier et al., 2012; Xifra-Porxas et al., 2019). Thus, it may be possible that age-related decrease in tactile sensibility and the ability to process sensory feedback (Cole et al., 1999) lead to declines in grip force control and increased brain activity within the motor-cortical network regardless of the task complexity. Following the compensation theory of aging (Reuter-Lorenz and Park, 2010), over-recruitment of cortical resources with aging appears to reflect a greater computational effort and oxygenation owing to compensatory patterns such as reorganization and redistribution of functional networks to counteract age-related structural and neurochemical changes in motor control systems (Bennett and Castiello, 1994; Mattay et al., 2002; Ward, 2006;

TABLE 1 | Correlations between relative oxygenated hemoglobin (Hboxy) and oscillatory beta activity.

ROI	EEG left PMC	EEG right PMC	EEG left SMC	EEG right SMC
Easy FTT—younger adults				
fNIRS left PMC	$r = 0.16$ ($p = 0.552$)	$r = 0.30$ ($p = 0.635$)	$r = 0.16$ ($p = 0.543$)	$r = 0.28$ ($p = 0.297$)
fNIRS right PMC	$r = 0.30$ ($p = 0.250$)	$r = 0.28$ ($p = 0.283$)	$r = 0.33$ ($p = 0.206$)	$r = 0.43$ ($p = 0.099$)
fNIRS left SMC	$r = 0.45$ ($p = 0.080$)	$r = 0.43$ ($p = 0.096$)	$r = 0.46$ ($p = 0.071$)	$r = 0.55$ ($p = 0.027$)*
fNIRS right SMC	$r = 0.37$ ($p = 0.160$)	$r = 0.34$ ($p = 0.197$)	$r = 0.37$ ($p = 0.151$)	$r = 0.37$ ($p = 0.062$)
Easy FTT—older adults				
fNIRS left PMC	$r = 0.41$ ($p = 0.110$)	$r = 0.46$ ($p = 0.076$)	$r = 0.43$ ($p = 0.098$)	$r = 0.26$ ($p = 0.335$)
fNIRS right PMC	$r = 0.44$ ($p = 0.087$)	$r = 0.47$ ($p = 0.065$)	$r = 0.48$ ($p = 0.083$)	$r = 0.26$ ($p = 0.325$)
fNIRS left SMC	$r = 0.47$ ($p = 0.069$)	$r = 0.49$ ($p = 0.055$)	$r = 0.47$ ($p = 0.066$)	$r = 0.25$ ($p = 0.351$)
fNIRS right SMC	$r = 0.48$ ($p = 0.062$)	$r = 0.50$ ($p = 0.048$)*	$r = 0.47$ ($p = 0.064$)	$r = 0.24$ ($p = 0.368$)
Complex FTT—younger adults				
fNIRS left PMC	$r = 0.12$ ($p = 0.661$)	$r = -0.14$ ($p = 0.615$)	$r = -0.20$ ($p = 0.662$)	$r = 0.12$ ($p = 0.964$)
fNIRS right PMC	$r = -0.07$ ($p = 0.791$)	$r = -0.09$ ($p = 0.730$)	$r = -0.34$ ($p = 0.888$)	$r = 0.07$ ($p = 0.800$)
fNIRS left SMC	$r = 0.12$ ($p = 0.654$)	$r = 0.09$ ($p = 0.730$)	$r = 0.15$ ($p = 0.590$)	$r = 0.25$ ($p = 0.348$)
fNIRS right SMC	$r = 0.01$ ($p = 0.963$)	$r = -0.18$ ($p = 0.948$)	$r = 0.03$ ($p = 0.914$)	$r = 0.16$ ($p = 0.554$)
Complex FTT—older adults				
fNIRS left PMC	$r = 0.33$ ($p = 0.207$)	$r = 0.36$ ($p = 0.166$)	$r = 0.36$ ($p = 0.176$)	$r = 0.27$ ($p = 0.310$)
fNIRS right PMC	$r = 0.36$ ($p = 0.172$)	$r = 0.40$ ($p = 0.122$)	$r = 0.39$ ($p = 0.141$)	$r = 0.30$ ($p = 0.265$)
fNIRS left SMC	$r = 0.37$ ($p = 0.157$)	$r = 0.36$ ($p = 0.169$)	$r = 0.38$ ($p = 0.148$)	$r = 0.25$ ($p = 0.358$)
fNIRS right SMC	$r = 0.40$ ($p = 0.126$)	$r = 0.38$ ($p = 0.138$)	$r = 0.40$ ($p = 0.134$)	$r = 0.25$ ($p = 0.361$)

Abbreviations: PMC, premotor cortex; SMC, sensorimotor cortex; r = Pearson correlation coefficient; Significance level $p < 0.05$.*

Noble et al., 2011; Larivière et al., 2019). Nevertheless, the results of deteriorations in grip force control and higher hemodynamic brain activity in older subjects cannot only be associated with the grip force tasks. As we did not include an additional control task such as a pinch force task or similar, increased brain activity in healthy aging during movements may be a general result that might also occur in other motor tasks and not only during the FTT. Furthermore, we identified decreases in beta power during both FTT relative to the resting period, known as MRBD, which showed the typical attenuation of beta activity during voluntary movements. After completion, beta power is pronounced, which has been interpreted as increased inhibition to maintain the status quo of the motor system (Pfurtscheller et al., 2006; Koelewijn et al., 2008; Engel and Fries, 2010). However, previous studies showed that older adults exhibited significantly higher beta oscillations at rest as well as greater MRBD during dynamic muscle contractions than did younger controls (Xifra-Porxas et al., 2019). However, our results did not show any significant differences in baseline beta power between the two groups. Additionally, older adults exhibited significantly greater MRBD in SMC bilaterally during the easy static FTT rather than during the complex variable FTT.

Second, all participants showed significantly higher inaccuracies in grip force control during the complex FTT than during the easy FTT. While the performance of grip force control was affected by task complexity, the respective hemodynamic activity seems to be unaffected. This is in line with the findings of previous studies, which showed that hemodynamic responses for all motor-cortical regions and two different grip force execution tasks (20% and 40% of the maximum grip strength) were similar (Wriessnegger et al., 2017). In contrast, significant differences in hemodynamic activity owing to task complexity were detected during finger tapping (Holper et al., 2009) and hand squeezing tasks in young healthy participants (Cramer et al., 2002). Thus, task-specific factors other than task complexity seem

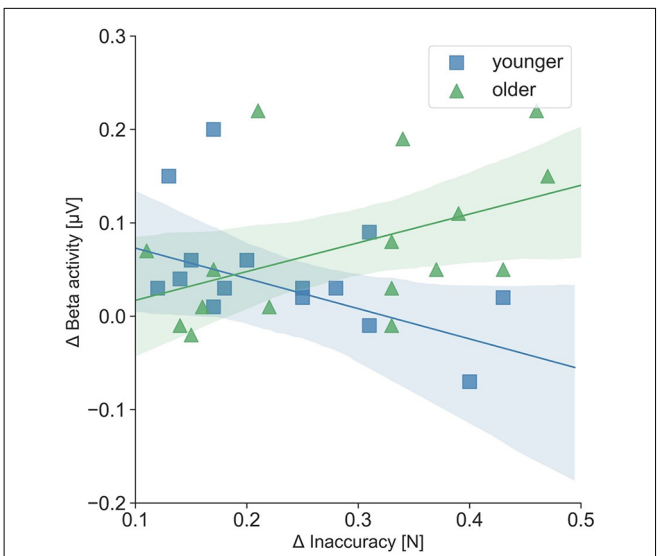

FIGURE 7 | Correlation of Δ inaccuracy in precise grip force regulation and Δ left PMC beta activity (μV); Δ = complex FTT − easy FTT; younger adults: r = − 0.32; older adults: $r = 0.46$. The correlations differed significantly between younger and older adults ($p = 0.03$).

to affect hemodynamic responses. Furthermore, we identified that complexity-related deteriorations in grip force control were correlated with increased left PMC beta activity in older adults, which differ significantly ($p = 0.03$) from the negative correlation of the younger adults. Therefore, the processing of visuomotor transformation and the integration of sensory feedback that underly precise grip force regulation (Hermsdörfer et al., 2003; Prodoehl et al., 2009) may be different between younger and older adults. In previous studies, older adults showed significantly higher absolute beta activity during movements than did younger adults (Heinrichs-Graham and

Wilson, 2016) that correlated with the degree of motor deficits during a visually guided grip task (Rossiter et al., 2014a). Due to the close relationship of beta activity and motor-cortical GABAergic inhibition (Rossiter et al., 2014b; Xifra-Porxas et al., 2019), it is suggested that age-related increases in beta activity may lead to a reduced ability to control the inhibition within the motor control network during movements. In the complex FTT, where the grip force must be regulated and adapted continuously, high cognitive effort in motor planning is required. Thus, older adults might show increased use of the PMC/SMA (Leff et al., 2011) which represents a key structure in the motor-cortical network driving manual movements by promoting and suppressing brain activity (Grefkes et al., 2008a; Bönstrup et al., 2016).

In addition to the age-related and task-complexity effects on grip force control and brain activity, correlations of oxygenated hemoglobin and oscillatory beta activity were found. During the easy FTT, increases in oscillatory beta activity were related to increases in oxygenated hemoglobin. However, in a previous study, increases in hemodynamic activity were accompanied by decreases of alpha and beta activity during a motor task (Lachert et al., 2017). Also, increases of alpha power following 10 and 20 Hz tACS were accompanied by decreases in oxygenated hemoglobin (Berger et al., 2018). Although it is well known that increases in oxygenated hemoglobin (Strangman et al., 2002) and suppressions in beta activity (Rossiter et al., 2014b; Xifra-Porxas et al., 2019) represent the neural correlated underlying movements, the relationship between the two parameters and how they change depending on motor tasks and complexities is not yet fully understood. Thus, numerous questions regarding the correlation of electrical signals and vascular changes (e.g., co-localization/time lag of the correlated changes, consistency of changes in connectivity) remain.

In summary, the older adults showed significant deterioration in grip force control and greater hemodynamic activity during both the FTTs than did the young adults, suggesting a greater computational effort and oxygen supply during the motor tasks (Ward, 2006) which represent a general age-related compensatory mechanism of changes in the sensorimotor network (Noble et al., 2011; Larivière et al., 2019). In contrast, increases in left PMC beta activity that are related to deteriorations in grip force regulation in older adults are task-complexity-dependent, thus representing an increased cognitive effort during motor planning or a compensatory mechanism of deficits in processing the sensory feedback within the sensorimotor network. Nevertheless, the age-related changes in oscillatory and hemodynamic activity as well as their correlation do not show clear results that allow an unequivocal conclusion. Understanding the relationship between motor control and brain activity as well as between motor recovery and its underlying neuroplasticity, remain major challenges in neurorehabilitation (Bönstrup et al., 2018). Thus, uncovering the link between age- and disease-related impairments of motor control and changes in brain activity to identify objective biomarkers for neuroplastic changes and motor recovery is a future goal in neurorehabilitation (Bönstrup et al., 2018; Coscia et al., 2019). Based on this, individualized training protocols and interventions with new technologies such as BMI and NIBS could be developed, which enable sustainable rehabilitation and optimization of the outcome and effectiveness of motor recovery (Semprini et al., 2018; Steinberg et al., 2019; Berger et al., 2019a,b).

Limitations

Although our findings regarding age- and task complexity-related changes in precise grip force regulation and brain activity were interesting, three limitations must be discussed and considered while designing future studies. First, the small number of subjects (n = 32) limits a clear and general conclusion. Referring to other neurophysiological studies (Noble et al., 2011; Xifra-Porxas et al., 2019), we focused on the methodological challenge of a combined EEG-fNIRS measurement during a motor task. In future studies, however, a higher sample size should be considered. Second, all participants were required to match the same target force in the visually guided FTT. Although our two groups do not differ in terms of MVC, previous studies have shown that grip strength declines with increasing age (Zammit et al., 2019) and after neurological diseases (Hermsdörfer et al., 2003). Thus, individual target forces depending on their own individual MVC can be considered in future FTT tasks to ensure that an optimal balance of support and challenge for each patient is achieved. However, different percentual forces ranging from 5 to 60% of the MVC were used in previous studies (Voelcker-Rehage and Alberts, 2005; Wriessnegger et al., 2017). Third, we had to focus on specific motor-cortical ROI because of the limited number of fNIRS channels and limited spatial resolution. Based on the knowledge that skilled grip force control depends on the intact communication of the entire sensorimotor network (Hermsdörfer et al., 2003) and because previous studies investigated motor-cortical areas using fNIRS during handgrip tasks (Wriessnegger et al., 2017), premotor and sensorimotor areas were chosen for investigation. Nevertheless, daily activities requiring visuomotor and sensorimotor integration (Vingerhoets, 2014) are based on a large neural network in which further cortical and subcortical areas are also involved (Vaillancourt et al., 2007; Wasson et al., 2010). Also, further analyses such as the coherence analysis between ROI to evaluate network communication and other recordings such as EMG on the performing arm to control muscle activity and to more precisely describe possible causes for age-related changes in grip force control would have added valuable further insights.

CONCLUSIONS

In this study, we found evidence for age-related changes in grip force control and hemodynamic activity as well as correlations between grip force control owing to task complexity and beta activity that were different in the left PMC between younger and older adults. Assessing both motor precision and brain activity is essential for understanding how motor control, oscillatory, and hemodynamic activity within the motor-cortical network are related. This knowledge could contribute to the development

of new therapeutic approaches to individualize and sustainably improve the treatment of motor impairments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local ethics committee of the Johannes Gutenberg-Universität Mainz. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB: conception and execution of the research project, data acquisition, statistical analysis, interpretation, and manuscript writing. AB acts as the corresponding author. FS, FT, and MD: conception and execution of the research project and review and critique of the manuscript. All authors have read and approved the final version of the manuscript.

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Abnormal Cingulum Bundle Induced by Type 2 Diabetes Mellitus: A Diffusion Tensor Tractography Study

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Purpose: In Type 2 diabetes (T2DM), white matter (WM) pathology has been suggested to play an important role in the etiology of T2DM-related cognitive impairment. This study aims to investigate the integrity of the cingulum bundle (CB), a major WM tract, in T2DM patients using diffusion tensor tractography.

Methods: Thirty-seven T2DM patients and 34 age-, sex- and education matched healthy controls were included and underwent diffusion tensor imaging. Tractography of bilateral CB tracts was performed and diffusion measurements were compared between the two groups. Next, brain regions with significant group differences on fractional anisotropy (FA) values were set as the region of interest (ROI), and the CB fibers that passed through were identified. Diffusion measures were extracted from these fibers to investigate their correlations with the cognitive performances and endocrine parameters.

Results: T2DM patients exhibited decreased FA in bilateral CB, increased mean diffusion (MD) in the right CB, and decreased length in the left CB. Through voxel-wise comparison, the most prominent FA difference was identified in the posterior segment of the CB and the reconstructed tract was part of the retrosplenial component. Importantly, the diffusion measurements of the tract were significantly correlated with the impaired performance in executive functioning and elevated insulin resistance (IR) in the T2DM group, instead of the control group.

Conclusions: The diffusion measurements in bilateral CB were altered in T2DM patients, which might reflect important neuropathologic changes in the fibers. Our study adds to knowledge about how the cingulum changes structurally along its entire length in T2DM and highlights the relationship between WM and cognitive performance. Besides, IR might be an important risk factor that warrants further exploration.

Keywords: Type 2 diabetes mellitus, cognitive impairment, cingulum bundle, diffusion tensor imaging, tractography, insulin resistance

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INTRODUCTION

Type 2 diabetes (T2DM) is a chronic metabolic disorder associated with a series of multi-systemic complications (American Diabetes Association, 2020). In recent years, impairment in cognitive functioning has been increasingly recognized in diabetic patients, the risk of dementia in which is about two times higher than the controls (Xia et al., 2020). Although the etiology remains controversial due to the complex comorbidities interplay, growing evidence suggests that vascular dysfunction, including both microvascular and macrovascular, might be an important risk factor (Reijmer et al., 2013). As revealed by brain imaging studies, hyperglycemia, and insulin resistance (IR) are related to increased white matter (WM) lesions and impaired WM connectivity (You et al., 2018). Therefore, WM pathology is suggested to play an important role in the etiology of T2DM-related cognitive impairment.

The cingulum bundle (CB) is a collection of fiber tracts longitudinally in the WM, interconnecting prefrontal, parietal, and medial temporal regions (Bubb et al., 2018). Given that these are core regions that have been repeatedly implicated in diverse processes, the CB is suggested to play a critical role in cognitive functioning, such as emotion and execution of motor- and attention-related tasks (Metzler-Baddeley et al., 2012; Bubb et al., 2018). Microstructural abnormalities in CB have been demonstrated in various conditions, including mild cognitive impairment (MCI), Alzheimer's disease (AD), depression, traumatic brain injury, and several other neuropsychological diseases (Kamagata et al., 2012; Metzler-Baddeley et al., 2012; Jang et al., 2013; Taylor et al., 2014). In T2DM patients, disruptions in major WM tracts have been consistently reported, which are possibly attributed to the increased vascular risk factors (Pruzin et al., 2018). Also, we and several other studies have reported disconnection in the default mode network (DMN), a key network to maintain the normal cognitive activities and its main components are linked by CB (van den Heuvel et al., 2008; Cui et al., 2015). Taken together, the critical connections and function of the CB prompted us to further characterize its profile and to elucidate the relationship between CB abnormality and the cognitive decline in T2DM patients.

DTI is a widely applied MR technique that is especially superior in imaging interconnecting WM tracts (Sanjari Moghaddam et al., 2019). It allows for the visualization of individual fiber bundles and quantification of its diffusion metrics, leading to a wide application in neurodegenerative diseases. Alteration in DTI indices is often independent of small vessel disease (SVD) revealed by conventional MRI, i.e., white matter hyperintensity (WMH) and lacunar infarcts, and precede the clinical symptoms (Reijmer et al., 2013). Altogether, DTI appears to be complementary to the classical MRI markers in detecting subtle WM changes at the very early stage of cognitive impairment. According to a recent review, most of the current T2DM-related DTI studies have surveyed whole-brain WM diffusion metrics using either voxel-based analyses (VBA) or tract-based spatial statistics (TBSS; Sanjari Moghaddam et al., 2019). Only one study specifically focused on the CB integrity, in which the tract was reconstructed using a region of interest (ROI) based approach, and only basic DTI measures including fractional anisotropy (FA) and mean diffusion (MD) were computed (Hoogenboom et al., 2014). A more detailed picture of the CB, including its volume, length, and density, and their correlation between cognitive and endocrine parameters, remains to be unraveled. The recently developed automatic streamline fiber tracking uses a track recognition based on a tractography atlas, with an additional algorithm to filter out false and unrelated tracks (Zhang et al., 2010; Yeh et al., 2019). It avoids the anatomical misplacement caused by manual tracing of apriori ROIs, thus providing better precision and quantification for the reconstruction.

In the current study, we aim to: (1) characterize the DTI indices of the CB in T2DM patients using the automatic streamline tractography, and compare them with healthy controls, (2) locate the most significant differences on the CB, and (3) explore the correlation between cingulum abnormalities with cognitive functioning and glycemic parameters. Our results would be promising in investigating the role of major WM tract disruption in the development of T2DM-related cognitive decline.

MATERIALS AND METHODS

Subjects

The study was approved by the institutional review board, and informed consent was obtained from all participants before evaluation. Participants were enrolled from the Department of Endocrinology or the local community through advertisement. All subjects were between 50 and 75 years old, with a minimum education of 6 years, and were group-matched in terms of age, sex, and education. Participants who met the following criteria were excluded: score on the Mini-Mental State Examination (MMSE) <24, score on the Hamilton Depression Rating Scale (HAM-D) ≥ 7, history of brain lesions such as tumor or stroke, and unrelated psychiatric or neurological disorder and MRI contraindications.

Diagnosis of T2DM was based on the American Diabetes Association criteria. All patients had a disease duration of at least 1 year and close self-monitoring. Neither a history of hypoglycemic episodes nor clinically detectable complications such as retinopathy, nephropathy, and peripheral neuropathy were reported. To exclude subjects in the pre-diabetes state, all healthy controls were performed with an oral glucose tolerance test (OGTT; 75 g dextrose monohydrate in 250 ml water). Subjects having a fasting blood glucose ≥ 7.0 mmol/L or postprandial glucose ≥ 7.8 mmol/L after 2 h OGTT were excluded.

Clinical Data Collection

A detailed questionnaire was used to collect information on medical histories and medication use. Weight, height, and waist circumferences were carefully recorded. Blood pressure was measured three times during the visit and averaged. Hypertension was defined as previously described. Blood samples

were collected *via* venipuncture at 7:00 AM after overnight fasting to measure the fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), fasting insulin, and cholesterol levels (i.e., triglyceride, total cholesterol, LDL cholesterol, and HDL cholesterol). Subsequently, blood samples were collected again at 9:00 AM, 2 h after OGTT for controls and a usual meal for patients, to measure the postprandial glucose. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to assess the degree of IR for all subjects except for those patients treated with insulin. This is because HOMA-IR may not accurately reflect insulin sensitivity in patients who require insulin or who have a minimal b-cell function.

Neuropsychological Tests

The general neuropsychological status of the participants was tested using MMSE. Subjects with a score below 24 were considered dementia and subsequently excluded, as described in the exclusion criteria. Tests that covered multiple domains were also performed, including the Auditory Verbal Learning Test (AVLT) for episodic memory, Complex Figure Test (CFT)-copy trial and Clock Drawing Test (CDT) for spatial processing ability, CFT-delay trail for spatial memory, Digit Span Test (DST) for working memory, Tail-Making Test (TMT) part A for attention and part B for executive functioning. All tests were performed in a fixed order by two experienced neurologists, which took about 60 min to complete.

MR Data Acquisition

Brain MRI data were acquired using a 3.0T MR-scanner (Siemens MAGNETOM Trio, Erlangen, Germany) with a 32-channel head coil. Foam padding and earplugs were used to reduce head motion and scanner noise. To obtain an anatomical reference, high-resolution T1-weighted imaging with a magnetization-prepared rapid gradient echo (MPRAGE) sequence was performed with a repetition time (TR)/echo time (TE) of 1,900/2.48 ms, a flip angle of 9°, an acquisition matrix of 256 × 256, a field of view (FOV) of 250 × 250 mm, and a slice thickness of 1 mm. DTI images were acquired by using the spin-echo echo-planar imaging (EPI) sequence (TR/TE = 10,000/95 ms, matrix size = 128 × 128, FOV = 256 × 256 mm^2, section thickness = 2 mm, 70 slices, 6/8 partial Fourier, NEX = 2, 30 gradient directions with a b-value of 1,000 s/mm^2 and one b = 0 s/mm^2 image. FLAIR images were also obtained for WMH evaluation: TR = 8,500 ms, TE = 94 ms, slice = 20, slice thickness = 5 mm, with each voxel size of 1.3 × 0.9 × 5 mm^3.

Evaluation of Small Vessel Disease

WMH and lacunar infarcts were assessed on fluid-attenuated inversion recovery images with a method described previously (Wahlund et al., 2001). To minimize the effects of WMH on fiber tracts, participants with a rating score >1 (confluence of lesions or diffuse involvement of each region) were excluded. The assessment was performed by two experienced radiologists blinded to the group allocations and a consensus was obtained through discussion between the raters.

To obtain the distribution of WMH and minimize its confounding effects on cingulum, FLAIR images were auto-segmented and binarized to obtain a WMH image using the Lesion Segmentation Toolbox version 3.0.0 implemented in SPM[1]. After normalizing into the MNI space, each individual's binarized WMH image was overlaid and divided by the number of subjects in each group to generate a probabilistic map in percentage. The voxel intensity value of the map indicates the frequency of WMH at each anatomical location. Therefore, the probabilistic map serves as a measure of inter-subject variability and WMH anatomical distribution. The overlap between the WMH and cingulum was assessed through visual inspection, and the probabilistic maps were compared between the T2DM and control groups to evaluate if there is a difference in WMH distribution.

Image Preprocessing and CB Tractography

The Diffusion MRI reconstruction and WM tractography were performed with DSI Studio software[2]. First, eddy current distortion correction was performed using the FSL toolbox[3]. To ensure the data accuracy, an automatic quality control routine was performed including b-table checking, calculation of the mean Pearson correlation coefficient of the neighboring diffusion-weighted images, and identification of slice-wise signal dropout for each slice in each diffusion-weighted image (Schilling et al., 2019). To obtain the spin distribution function, the diffusion images were reconstructed in the MNI space using q-space diffeomorphic reconstruction, as described previously (Yeh and Tseng, 2011). The diffusion sampling length ratio was set at 1.25 and the average dataset was resampled to 2-mm isotropic resolution. The restricted diffusion was quantified using restricted diffusion imaging (Yeh et al., 2017).

Subsequently, automatic fiber tracking based on atlas-guided track recognition was performed with DSI studio software and false tracks and unrelated tracks were filtered out based on a previously reported atlas (Yeh et al., 2018). The streamline tractography was generated using the following parameters: seeds = 1,000,000, anisotropy threshold = 0.1, angular threshold = 60°, step size = 0.5 mm. The fiber trajectories were then smoothed by averaging the propagation direction with 80% of the previous direction. Fibers shorter than 30 mm or longer than 250 mm were discarded. Finally, topology-informed pruning was applied to further remove the false connections (Yeh et al., 2019).

After obtaining the reconstructed data, the left and right CB were identified according to the HCP tractography atlas (Yeh et al., 2018). To quantify the microstructural abnormalities, we computed several DTI measures, including the number, mean length, volume, surface area, FA, and MD values of the tracks, which were included in the group comparison.

Voxel-Based Analysis and ROI-Based Tractography

To further identify the most prominently affected regions in the impaired CB, we performed a voxel-based group comparison

[1] www.statistical-modelling.de/lst.html
[2] http://dsi-studio.labsolver.org/
[3] http://www.fmrib.ox.ac.uk/fsl/

on the FA maps. The $P_{uncorrected}$ value was set at 0.005, with a cluster size of 26 voxels (determined *via* Monte Carlo simulation) corresponding to a $P_{corrected}$ of 0.05. The identified clusters with significant FA differences were then set as the ROIs and the CB fibers that passed through were retained. Diffusion measures of the fibers were then extracted to investigate their group differences and correlations with the clinical parameters.

Statistical Analysis

Demographic variables and cognitive performance were compared between the two groups using SPSS software (Version 21.0, SPSS Inc., Chicago, IL, USA). Normal distributions were tested using the Kolmogorov–Smirnov test. An independent two-sample *t*-test was used for continuous variables, a nonparametric Mann–Whitney *U* test, for asymmetrically distributed variables, and a χ^2 test, for proportions. *P*-values <0.05 were considered statistically significant.

Diffusion values of bilateral CB were compared between groups using multivariate ANCOVA, controlling for age, gender, education level, and WMH volume to minimize their confounding effects. Given that the CB has distinct segments and their according functions, we assumed that to take the CB as a whole would overlook/underestimate its correlation with the clinical parameters. Therefore, the correlations among DTI measures, cognitive performance, and endocrine metrics (plasma glucose and HbA1c levels, HOMA-IR, and disease duration) were performed based on the tract reconstructed to form the ROIs with group difference. Partial Pearson correlation analyses were performed, adjusted for the same covariates as those controlled in the two-sample *t*-tests. A *P*-value <0.05 was considered statistically significant.

RESULTS

Demographic and Neuropsychological Results

A total of 40 T2DM patients and 43 HCs were recruited. Three HCs quitted the cognitive assessment and two HCs refused the blood collection. Two patients and one HC were excluded due to extensive WM lesions. One patient and three HCs were further excluded after the quality control routines for DTI images. Therefore, 37 T2DM patients and 34 healthy controls were included in the final analyses. Clinical and demographic characteristics for the diabetic and control groups are summarized in **Table 1**. No significant differences were found between groups in terms of age, education, gender distribution, WMH load, blood lipid, and blood pressure. Although BMI is higher in the diabetic group, the difference did not reach statistical significance. As expected, fasting glucose, postprandial glucose, HbA1c, and IR were all significantly elevated in the patients' group.

Cognitive results are summarized in **Table 2**. Both groups performed within the normal range on MMSE, but the diabetic group scored lower than the control group on the CFT-Delay trial and TMT-part B. These tests covered cognitive domains including memory, information processing, and executive functioning, which are all frequently reported to be impaired in diabetic subjects. Notably, disease duration was exclusively correlated with the score on CFT-Delay ($R = -0.422$, $P = 0.009$). No additional correlations were found among other clinical variables.

TABLE 1 | Demographics and clinical variables of the study sample.

Measures	T2DM (*n* = 37)	Control (*n* = 34)	*P*-value
Age (years)	59.7 ± 7.3	56.9 ± 6.1	0.08
Sex (male/female)[a]	19/18	12/22	0.23
Education (years)	9.9 ± 3.3	10.4 ± 2.4	0.49
Diabetes duration (years)	8.2 ± 4.5	-	-
Insulin treatment (n)	10	-	-
HbA1c (%, mmol/mol)	7.8 ± 1.5	5.6 ± 0.3	**<0.001***
FPG (mmol/L)	7.7 ± 2.2	5.5 ± 0.4	**<0.001***
Postprandial glucose	15.1 ± 4.8	6.4 ± 1.8	**<0.001***
HOMA-IR	3.5 ± 2.0	2.6 ± 1.4	**0.03***
BMI (kg/m^2)	24.5 ± 3.1	23.4 ± 2.8	0.15
Systolic BP (mmHg)	135.7 ± 14.8	129.8 ± 12.4	0.07
Diastolic BP (mmHg)	85.0 ± 10.9	85.6 ± 11.5	0.85
Total cholesterol (mmol/L)	5.3 ± 1.2	5.0 ± 0.8	0.64
Triglyceride (mmol/L)	1.5 ± 0.8	1.3 ± 0.7	0.37
HDL cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.3	0.55
LDL cholesterol (mmol/L)	3.2 ± 0.8	3.1 ± 0.6	0.44
White matter lesions (range)	0–6	0–7	0.35
Lacunar infarcts (n)[a]	7	4	0.52

*Data are represented as mean ± (SD), n, or range. [a]Statistical analyses were performed by χ^2 test. *P-value < 0.05. FPG, fasting plasma glucose; HOMA-IR, homeostasis model assessment of insulin resistance; BMI, body mass index; BP, blood pressure. Significant values in bold.*

Distribution of WMH

The color-coded composite probability map in MNI anatomic space for all participants is shown in **Figure 1**. Visual inspection of the map suggested a predominant WMH distribution for periventricular and deep WM areas, especially in frontal periventricular regions (color in red). The blue color displays the complete CB derived from the fiber template HCP1021-1mm[4], which is largely independent of the surrounding WMH. Comparison of the probability map between the T2DM and HC groups also suggested a similar distribution, with the most prominent WMH in identical regions. Taken together, the above results demonstrated the little effect of WMH on the integrity of the CB.

Tractography of the Cingulum

Figure 2 shows the reconstructed CB of four representative subjects. As a symmetrical sickle-shaped bundle that encircled the CC, the shape and trajectory of CB are highly consistent with the WM template and previous literature. However, the frontal fibers were less than the template, which might be attributed to normal aging (Sibilia et al., 2017). Through visual inspection, the fibers in the CB in diabetic representatives were slender and more diffusely orientated compared with the healthy controls.

[4]https://pitt.app.box.com/v/HCP1021-1mm

TABLE 2 | Cognitive performance of Type 2 diabetes (T2DM) and control groups.

Measures	T2DM (n = 37)	Control (n = 34)	P-value
MMSE	28.4 ± 1.2	28.9 ± 1.1	0.11
AVLT-copy	6.1 ± 1.2	6.2 ± 1.4	0.84
AVLT-delay	6.0 ± 2.3	6.2 ± 2.3	0.78
CFT-copy	34.5 ± 1.7	35.0 ± 1.4	0.22
CFT-delay	14.3 ± 5.9	17.7 ± 6.0	**0.02***
DST (forward)	7.0 ± 1.2	7.4 ± 1.6	0.21
DST (backward)	4.2 ± 0.9	4.6 ± 1.5	0.15
TMT-part A	65.6 ± 21.5	61.8 ± 14.8	0.40
TMT-part B	176.2 ± 61.4	147.8 ± 45.3	**0.03***
CDT	3.3 ± 0.6	3.5 ± 0.5	0.17

*Data are represented as mean ± (SD). *P-value < 0.05. MMSE, mini-mental state examination; AVLT, auditory verbal learning test; CFT, complex figure test; DST, digit span test; TMT, trail-making test; CDT, clock drawing test. Significant values in bold.*

Between-Group Differences of DTI Metrics

The DTI results are summarized in **Table 3** and **Figure 3**. After controlling for age, gender, education level, and WMH scores, we observed a significant between-group effect for the DTI metrics of bilateral CB tracts. Patients exhibited decreased FA (P = 0.030) and increased MD (P = 0.024) in the right CB. Meanwhile, lower FA (P = 0.003) and shorter fiber length (P = 0.042) in the left CB were also identified in the diabetic group. Although patients also showed higher MD in the left CB (P = 0.016), the difference was insignificant after introducing the covariates (P = 0.180). Of further note, none of the tract-number, volume, and surface areas were different between the groups, but there was a trend of fewer fibers and smaller tracts in both bilateral CB in the diabetic group.

The VBA revealed that the most prominent group difference was located in the posterior part of the right CB, as shown in **Figure 4** (color in red). The reconstructed tract is shown in yellow, which belongs to the dorsal segment of the CB, connecting the medial frontal and parietal lobes along the cingulate cortices. Nevertheless, all the diffusion measurements of the tract did not differ between the two groups.

Correlational Analyses

Correlational results are shown in **Figures 4A–D**. In T2DM group, the time spent in TMT-B was negatively correlated with FA (R = −0.377, P = 0.037), volume (R = −0.446, P = 0.012), and surface area (R = −0.493, P = 0.005) of the reconstructed tract. Noteworthy is that the FA, volume, and surface area were significantly correlated with each other (all P-values < 0.01). Due to the significant effects of disease duration on the CFT-Delay trial, we further included disease duration as a covariate in the partial correlation analyses. However, the performance was not correlated with any of the diffusion measures. Among all the endocrine variables, IR was the only one that correlated with the diffusion measures of tract length (R = −0.436, P = 0.042).

DISCUSSION

In the current study, we characterized the microstructure of bilateral CB and its relationship with cognitive performance and endocrine measurements. Using fiber tractography, we demonstrated reduced FA, increased MD, shorter fibers, and a trend of the smaller tract in the CB in diabetic patients, which were independent of WM lesions. Most importantly, the impairments in the CB were significantly correlated with the worse performance in the TMT-B and elevated IR in the patients' group.

Several existing studies using different modes of DTI analyses have consistently reported diffusion abnormalities in the CB in T2DM patients (Sanjari Moghaddam et al., 2019). Most of them have surveyed the whole brain relying on either VBA

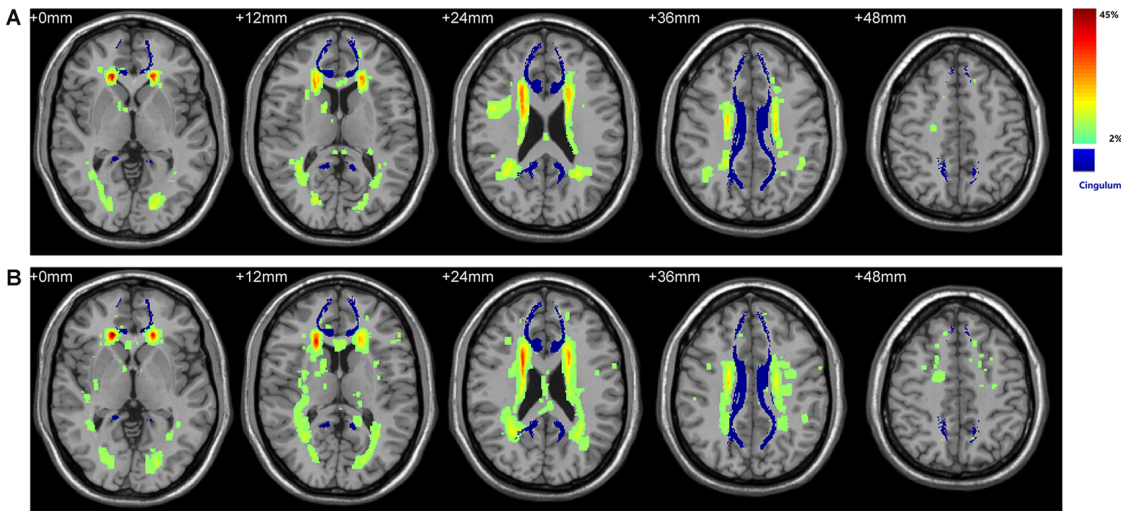

FIGURE 1 | Spatial distribution of WMH and its overlay with bilateral cingulum. **(A)** WMH distribution in the control group. **(B)** WMH distribution in the Type 2 diabetes (T2DM) group. Color denotes the frequency of WMH at each anatomical location, serving as a measure of inter-subject variability in each group. The WHM distribution has a predilection for periventricular and deep WM areas, especially in frontal periventricular regions (red). Bilateral CB shown in blue was derived from the fiber template HCP1021-1mm (https://pitt.app.box.com/v/HCP1021-1mm). WMH, white matter hyperintensity; WM, white matter; CB, cingulum bundle.

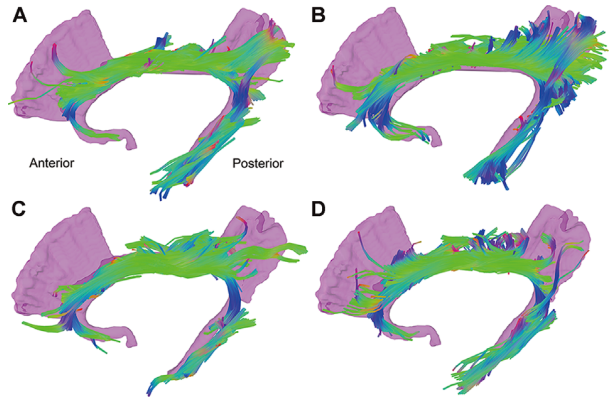

FIGURE 2 | Tractography results of the CB of four representative subjects. The reconstructed CB is a symmetrical sickle-shaped bundle. The fibers in the CB in diabetic representatives **(C,D)** were slender and more diffusely orientated compared with the healthy controls **(A,B)**. CB, cingulum bundle; CC, corpus callosum.

or TBSS method, providing results limited to regional clusters instead of treating the tract as a whole. The only study known to us that used the tractography method was based on manually drawn ROIs, which would be prone to anatomical misplacement (Hoogenboom et al., 2014). In comparison, the atlas-guided automatic tract reconstruction in the present study enabled us to clearly depict the CB in its entirety. Without the need for extensive anatomical experience and high reproducibility of ROI placement, such an approach could minimize false-positive tracing and prominently improve the stability of the diffusion measurements (Reich et al., 2010). Taken together, we consider our results to be more CB-specific and reproducible than the existing literature.

Our results indicated that both FA and MD are significantly differed between the two groups, especially when there was little overlay between these abnormalities and the WMH revealed by conventional MRI. These results suggested that the cingulum abnormalities were independent of WMH, while DTI is clearly complementary to traditional MRI in detecting subtle WM abnormalities. As a core part of the limbic system (Dalgleish, 2004), CB has complex connections and multiple functions, being consistently reported to be injured in a wide range of neurological and psychiatric disorders. The disruption of CB in T2DM is thus not unexpected, which might reflect an overlapping pattern during the process of cognitive decline. Besides, we and several other studies have previously reported DMN disconnection in T2DM patients, especially in its posterior components (Cui et al., 2015; Ishibashi et al., 2018; Liu et al., 2019). Given that CB is the anatomical basis for DMN (van den Heuvel et al., 2008), the present CB abnormalities not only are supported by the previous functional results but also extend fMRI studies by adding anatomical evidence. Nonetheless, our results revealed no difference in the fiber number, volume, or surface area, which might be attributed to the large individual variance, as shown by the SD values in the results.

The disrupted CB may be associated with pathologic processes of T2DM. As two representative diffusion metrics, FA indicates the degree of anisotropic movement of water molecules, while MD measures the average movement in three possible dimensional directions (Alexander et al., 2007). Briefly, the reduced FA and increased MD, as shown in our results, are indicative of lower microstructural integrity within the CB. Similarly, lower FA and higher MD values have been frequently reported in T2DM patients in diffuse WM tracts, although the regions tend to differ across studies (Hoogenboom et al., 2014; Raffield et al., 2016; Yoon et al., 2017). Diabetes is often associated with an increased burden of SVD, leading to WM impairment that is sensitive and susceptible to ischemic stress (Hardigan et al., 2016). A recent clinical study also suggested diabetes as an important risk factor for WMH progression (Tamura and Araki, 2015). Although the changes in diffusion parameters might reflect myelin or axon pathology according to their physiological nature (Alexander et al., 2007), the specific neuropathology remains unproved and merits further exploration.

VBA suggested that the disruption has a predilection for the posterior CB segment. According to anatomical reviews,

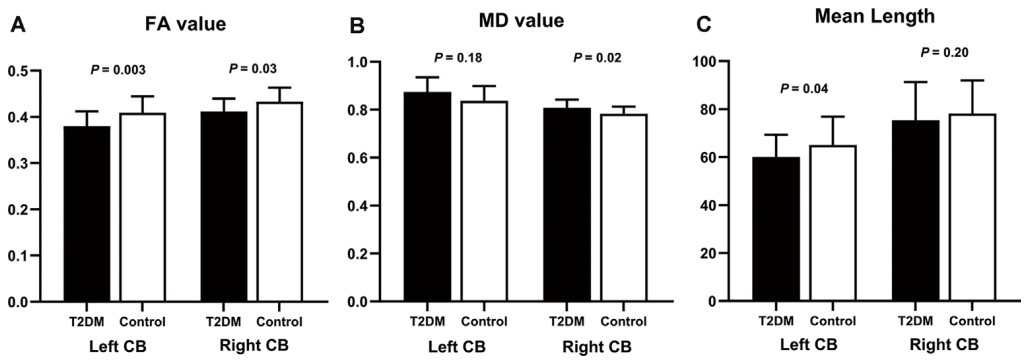

FIGURE 3 | Group differences of diffusion measurements in bilateral CB. The comparison used age, sex, education, and WMH as covariates. **(A)** In the T2DM group, FA values were significantly decreased in bilateral CB. **(B)** MD values were higher in the T2DM group, but the significance in the left CB did not remain after controlling for the covariates. **(C)** T2DM patients had shorter fiber length compared to the controls, but only the difference in the left CB was significant after introducing the covariates. CB, cingulum bundle; FA, fractional anisotropy; MD, mean diffusivity. The error bars represent the standard deviation.

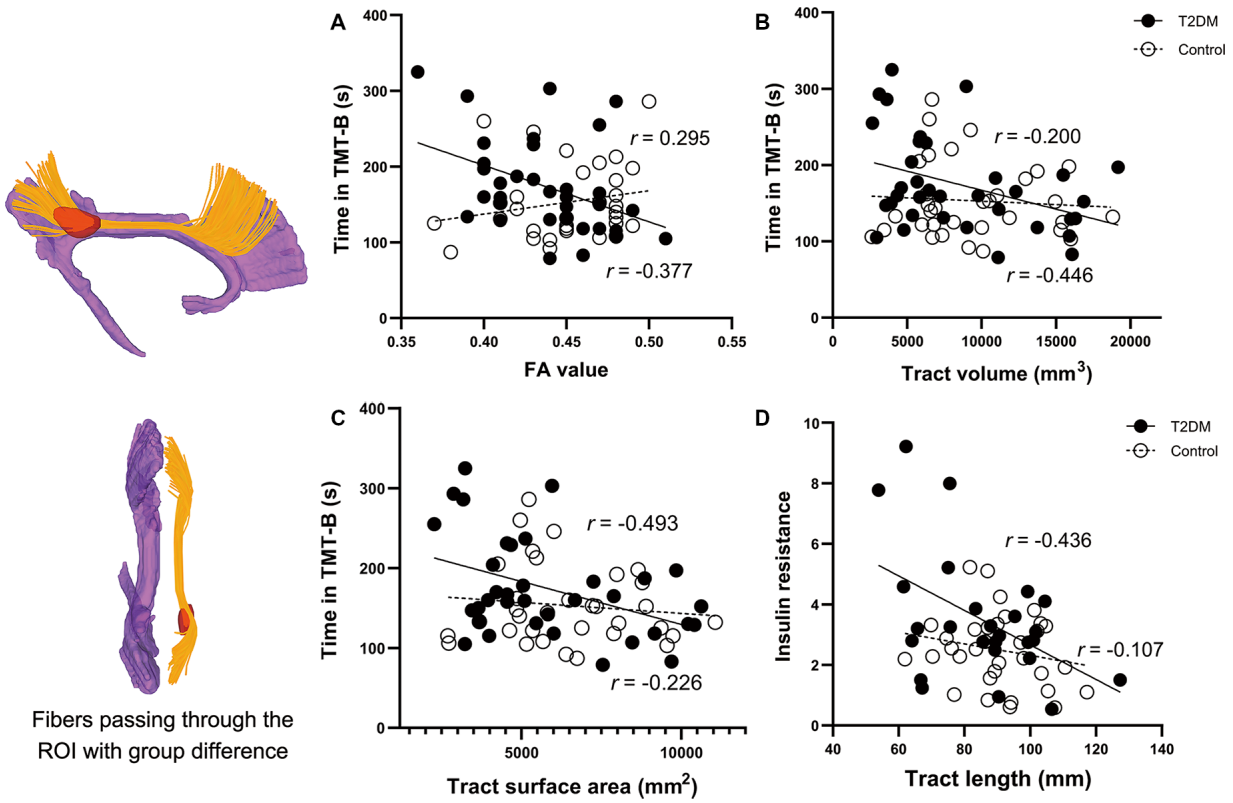

FIGURE 4 | Correlations between the clinical parameters and the diffusion measures of the reconstructed tract using ROI-based tractography. Figures on the left are the results of the voxel-wise comparison of FA maps between the two groups. Significantly decreased FA was found in the posterior part of the right cingulum (the region in red). The tract passing through the ROI is illustrated in yellow. Correlations in the T2DM group are presented in black circles, while correlations in the control group are presented in white circles. **(A–C)** In the T2DM group, the time spent in TMT-B was negatively correlated with FA, volume, and surface area of the tract. **(D)** IR in the T2DM group was negatively correlated with the mean length of the reconstructed tract. No such correlations were observed in the control group. The CB shown in purple was derived from the template HCP1021-1mm (https://pitt.app.box.com/v/HCP1021-1mm). CFT-delay, complex figure test-delay trial; TMT-B, trail making test-part B; IR, insulin resistance; FA, fractional anisotropy; ROI, the region of interest.

TABLE 3 | Diffusion measures of bilateral cingulum bundle in both groups.

	Measures	T2DM (*n* = 37)	Control (*n* = 34)	*P*-value
Left CB	Tract number	2,331.7 ± 1,259.3	2,362.4 ± 1,151.8	0.74
	Mean length	60.1 ± 9.3	65.1 ± 11.8	**0.04***
	Volume	12,695.6 ± 4,461.9	13,371.6 ± 3,942.4	0.37
	Surface area	8,186.7 ± 2,067.7	8,551.2 ± 1,847.4	0.31
	FA	0.38 ± 0.03	0.41 ± 0.04	**0.003***
	MD	0.87 ± 0.62	0.84 ± 0.61	0.18
Right CB	Tract number	1,944.8 ± 1,271.0	1,988.6 ± 986.0	0.87
	Mean length	75.4 ± 15.9	78.2 ± 13.7	0.23
	Volume	12,190.3 ± 5,389.4	12,873.2 ± 4,013.4	0.15
	Surface area	7,847.2 ± 2,473.9	8,246.9 ± 1,930.6	0.13
	FA	0.41 ± 0.28	0.43 ± 0.30	**0.03***
	MD	0.81 ± 0.03	0.78 ± 0.03	**0.02***

*Data are represented as mean ± (SD). *P-value <0.05. The comparison was performed after controlling for age, sex, education, and WMH. CB, cingulum bundle; FA, fractional anisotropy; MD, mean diffusion. Significant values in bold.*

the CB can be at least divided into the dorsal and ventral components, each having distinct connections and functions, although how best to subdivide the cingulum is still under debate (Bubb et al., 2018). The present cluster with significant group difference lies in the posterior part of the dorsal CB, i.e., the "retrosplenial" component, which is closest anatomically to the posterior cingulate cortex (PCC) and connects the medial frontal and parietal lobes along the cingulate cortices (Jones et al., 2013). As core components of the well-defined DMN, the dysfunction of PCC has been observed in various neurodegenerative conditions

and is considered hallmarks of cognitive decline (Greicius et al., 2004). Similarly, both volumetric, metabolic, and perfusion reduction in the PCC region have been reported in T2DM patients (Baker et al., 2011; Cui et al., 2017). Given the close anatomical connections, it is not unexpected to detect the abnormalities in the retrosplenial component. Interestingly, reduced FA in posterior CB is a consistent finding in MCI and AD (Metzler-Baddeley et al., 2012), in contrast to normal aging that mainly involves the anterior component (Sibilia et al., 2017). T2DM is an established risk factor for developing all-cause dementia, yet the link is mediated through vascular dysfunction, AD neuropathologic changes, or both, remain debated (Pruzin et al., 2018). Although our results suggested comparable CB abnormalities in T2DM and AD patients, whether T2DM is more likely to develop AD pathologies needs more evidence.

Several diffusion measures of the reconstructed tract that pass through the group-differed cluster were exclusively correlated with the performance in TMT-B. As mentioned above, the tract belongs to the retrosplenial segment, the integrity of which is associated with not only memory but also visuospatial processing and executive functions (Vogt et al., 1995), which covered the main cognitive domains that are required in the successful performance of the TMT-B (Chen et al., 2009). Therefore, the decline in executive functioning, which is one of the key manifestations in T2DM-related cognitive impairment, might be attributed to the disruption in CB integrity. On the other hand, IR rather than the glucose level was the only endocrine parameters correlated with the diffusion measures. IR, the central pathological feature of T2DM, is hypothesized to interfere with normal synaptic transmission and plasticity, which may be implicated as part of the pathogenic process of dementia (Ferrario and Reagan, 2018). Based on our results, we speculated that IR might be an important risk factor for developing T2DM-related cognitive impairment. The insignificant correlation between glucose level and DTI measurements might be interpreted as the glucose fluctuations and the effects of various medications.

Several limitations of our study need to be addressed. First, the small sample size may have limited the detection of group differences and our interpretation of the current results. Second, the current results might be confounded by the presence of T2DM-related complications and comorbidities. However, we did control for the possible risk factors, which should have minimized the confounding effects. Third, most of our patients and some HC were taking various medications. Further studies should include more participants and to test the effects of interventions. Finally, there are deficits in the specificity and precision of the DTI metrics, especially when the targeted areas have complex crossing fibers. More advanced imaging methodologies such as diffusion spectrum and kurtosis imaging should be applied, and more WM tracts should be investigated using multi-modality design.

Our findings in T2DM patients showed significant alterations in bilateral CB, while the most prominent difference was found in the right retrosplenial segment. More importantly, the diffusion metrics of the reconstructed tract passing the group-differed cluster were significantly correlated with cognitive performance and IR level. These results not only addressed the role of disrupted major WM tract in the process of T2DM-related cognitive decline but also highlighted IR as an important risk factor. However, the underlying pathophysiology of these microstructural changes should be examined in future studies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IEC for Clinical Research, Zhongda Hospital, Southeast University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SJ conceived and designed the experiments. YCu and T-YT collected and analyzed the MR data, and wrote, reviewed, and edited the manuscript. C-QL, YCa, and TL contributed to data quality control and data analyses. Y-CW and G-JT reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

SYSTEMATIC REVIEW
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The Cognitive Profile of Mild Cognitive Impairment Due to Dementia With Lewy Bodies—An Updated Review

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Objective: Dementia with Lewy Bodies (DLB) is the second most common type of neurodegenerative dementia. Yet, the domain-specific cognitive impairment of the mild cognitive impairment (MCI) phase of this disease (DLB-MCI) is still not been established. This article gives an updated review on the neuropsychological profile of DLB-MCI, building on the findings from a previous review.

Methods: We performed systematic review and searched five different electronic databases (Scopus, Cochrane, EMBASE, MEDLINE, and PsycINFO) in May 2020 based on a PICO scheme. Our search was then restricted to articles published in 2019 and 2020. Ending up with a total of 90 articles to be reviewed by abstract and/or full text.

Results: In total four papers were included, whereof only one met our full inclusion criteria. Despite a substantial heterogeneity, our findings indicate that DLB-MCI patients have a pattern of executive, visuospatial, and attentional deficits.

Conclusion: The findings indicate that the neuropsychological profile of DLB-MCI is characterized by executive, visuospatial, and attentional deficits. Furthermore, the shortage of studies clearly underlines the paucity of published research into DLB-MCI and emphasizes the need for well-controlled studies.

Keywords: dementia with lewy bodies (DLB), mild cognitive impaiment, cognition, predementia, neuropsychological profiles, prodromal DLB

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INTRODUCTION

Dementia with Lewy bodies (DLB) comprises up to 24% of all neurodegenerative dementia cases (Hogan et al., 2016). It is thereby the second most prevalent type of neurodegenerative dementia, only exceeded by Alzheimer's Disease (AD), and the need for accurate and early diagnostic identification is thus of great social importance. DLB is characterized by dementia in combination with different core clinical features of rapid eye movement (REM) behavior disorder (RBD), parkinsonism, cognitive and alertness fluctuations, and visual hallucinations. In addition, there are a number of supportive clinical features, indicative biomarkers, and supportive biomarkers to be used when establishing a clinical diagnosis (McKeith et al., 2017).

Both Parkinson's disease (PD) with and without dementia (PDD) and DLB are thought to be on separate ends of a continuum of Lewy Body diseases (LBDs) (Aarsland et al., 2009). Thus, the two diagnoses share important pathophysiological and clinical features, including motor, neuropsychiatric, cognitive, and autonomic symptoms. Because of this extensive overlap, it has even been up for discussion whether PDD and DLB really are two distinct diagnoses (McKeith, 2009; Berg et al., 2014). Today DLB is diagnosed when dementia precedes or accompanies parkinsonism, whereas PDD is diagnosed when dementia occurs after PD onset. For research matters, the differentiation of DLB and PDD is defined by a 1-year criterion, where PDD is diagnosed when PD occurs a year or more before dementia (McKeith et al., 2017).

Moreover, studies also suggest that both neuropsychological deficits and core clinical features arises early in the disease progression (Sperling et al., 2011; Donaghy et al., 2015), and the phase preceding dementia has therefore been heavily investigated over the last years. This phase, called the mild cognitive impairment (MCI) stage, points to the intermediate stage between normal cognitive aging and dementia. Here objective cognitive decline is present, but not to the extent that it interferes with functional daily living. Research now suggests that the MCI stage due to the different dementia types have their unique cognitive profiles equal to their associated dementias. As of today, the MCI stage for both AD (AD-MCI) and PDD (PD-MCI) are defined (Albert et al., 2011; Litvan et al., 2012), but the proposed MCI stage for DLB (DLB-MCI) (Donaghy et al., 2018) is still lacking support from clinical studies. Indeed, research criteria for the diagnosis of prodromal DLB has only recently been published (McKeith et al., 2020), defining DLB-MCI as MCI with concurrent probable or possible DLB (McKeith et al., 2017, 2020).

Furthermore, the need for validation of the DLB-MCI criteria is especially pressing, as there is an overall trend in the field of dementia to advance the point of diagnosis to the MCI stage. The high rate of misdiagnosis, the symptomatic overlap between different diagnoses, and the fact that the different diagnoses calls for different treatment makes the validation even more exigent. Moreover, defining the cognitive profile of DLB-MCI might also be of important prognostic value, as neuropsychological tests is shown to be good predictors for conversion from MCI to AD (Belleville et al., 2017).

A recent review indicates that the cognitive profile of DLB-MCI is characterized by executive dysfunction, slow processing speed, in addition to visuospatial and working memory deficits (Ciafone et al., 2020). It thus resembles the cognitive profile for the PD-MCI. However, the authors emphasized the paucity of research into DLB-MCI and therefore the need for more studies. Given both the time passed since Donaghy et al. (2018) proposed a DLB-MCI and the importance of the area, one might expect that more research is now available.

METHODS

This is an updated and systematic review of the evidence base underlying the domain-specific cognitive impairment associated with DLB-MCI, building on a recently published review based on a search performed in January 2019 (Ciafone et al., 2020). This systematic review conforms to the PRISMA reporting guidelines.

Search Strategy

Building on a PICO scheme (**Supplementary Material**), we searched Scopus, Cochrane, EMBASE, MEDLINE, and PsycINFO databases in May 2020 (2020-05-14 and 2020-05-22). Free text words were used in all databases: "Lewy" or "DLB" with "dementia" in combination with "prodromal*" and "cognit* dysfunction/decline/impair*/defect*/deficit*," MCI and/or SCD [subjective cognitive decline]. In addition to text words, medical subject headings from thesaurus were used when possible, thus in Medline and Cochrane Central (Lewy Body disease, prodromal symptoms, cognitive dysfunction), EMBASE (prodromal symptoms, diffuse Lewy body disease, dementia assessment, cognitive defect), and PsycINFO (dementia with Lewy bodies, prodrome, cognitive impairment). The search was restricted to title, abstract, and keyword. The search was performed in collaboration with a University librarian.

Inclusion and Exclusion Criteria

We included studies that measured domain specific cognitive functioning in DLB-MCI. Both studies that reported scores on isolated neuropsychological tests as well as those including domain specific composite scores were accepted. On the other hand, our exclusion criteria were $n < 10$, lack of healthy control (HC) subjects, unclear diagnostic and MCI definitions, and use of global cognitive composite scores. That is, studies who only reported cognition in terms of global Mini-Mental Status Examination (MMSE) or Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005) scores were excluded, as opposed to those who for example reported domain-specific MoCA subscores. Furthermore, studies including only one case of DLB-MCI in addition to other MCI groups were considered case studies, and thus removed.

RESULTS

The search yielded a total of 10,276 references: 6,300 after removing duplicates using EndNote and 5,747 after manually removing remaining duplicates. These references were further restricted to those published in 2019 and 2020, resulting in a total of 684 references subjected for title screening. After a gross title screen, removing animal studies, articles with a flagrantly irrelevant topic or articles that were not published in English journals, 89 references remained to be reviewed by abstract. See **Figure 1**.

The abstract review typically removed case studies, and articles that did not target DLB did not include cognitive measures or only included global cognitive composite scores (such as MMSE or MoCA total score). The main reasons for removal after full text revision were studies that did not distinguish between prodromal symptoms and symptoms of (mild) dementia, unclear neuropsychological data, and lack of control groups. Reviews, meta-analyses, abstracts, posters, discussion papers, and commentaries were also screened out

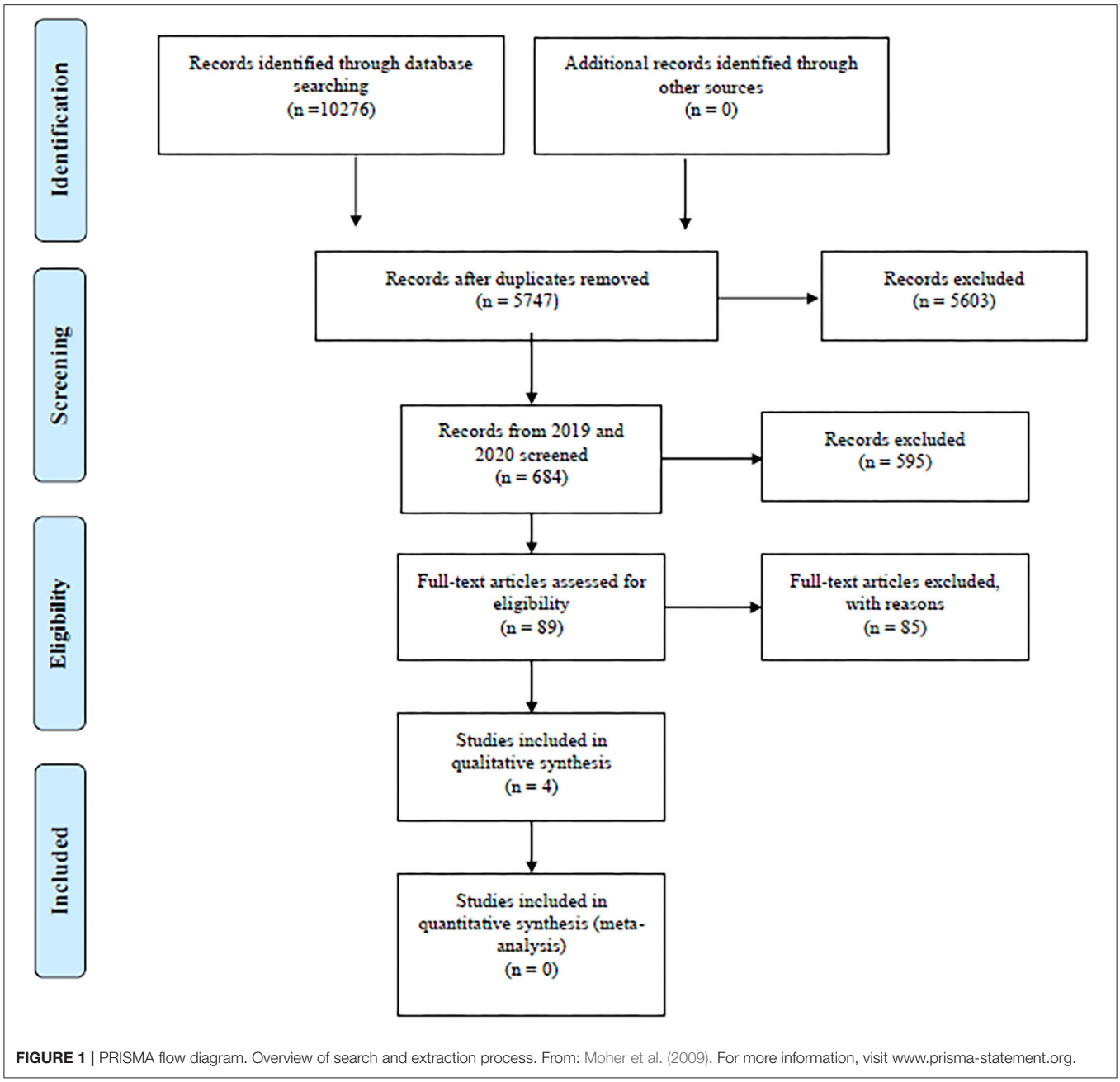

FIGURE 1 | PRISMA flow diagram. Overview of search and extraction process. From: Moher et al. (2009). For more information, visit www.prisma-statement.org.

during the process. At both the point of abstract review and full text review, ambiguous cases were discussed between the main authors (MSH, MHB) until consensus was achieved. We extracted baseline data in longitudinal studies, unless otherwise stated.

Since only one study met the full inclusion criteria (Massa et al., 2019), we *post hoc* chose to include studies that did not have HCs and had unclear diagnostic criteria. Doing this we ended up with a total of four articles for this review (Massa et al., 2019; Unger et al., 2019; van de Beek et al., 2020; Yoo et al., 2020). See **Table 1**.

Synthetisized Findings

The only study that met the full inclusion criteria was a longitudinal study by Massa et al. (2019). The DLB-MCI patients were diagnosed with MCI prior to PD, with a mean time of 2.9 ± 1.9 years (range 0.5–6.1). These patients were then matched and compared to both HCs and cognitively normal PD patients. However, they only used cognitively normal PD patients instead of HCs in the analysis of cognitive data. They report heterogeneous findings at the baseline, with the cognitive profiles of DLB-MCI both being amnestic, non-amnestic and single- or multidomain. They did nevertheless find

TABLE 1 | Overview of the included studies.

References	Country	Study type (time of inclusion)	Study aim	Patient group (n)	Control group (n)	MCI criteria	Diagnostic criteria	Neuropsychological tests	Findings: impaired functions in the patient group
Massa et al., 2019	Italy	Longitudinal follow-up study (2004–2017)	To characterize the neuroimaging and neuropsychological characteristics of LBD-MCI	MCI-P (13).	HC (18) PD-MOT (11)	Impairment on ≥ two NP tests, preserved ADL/IADL, CDR = 0.5	PD as defined by Gelb et al. (1999)	MMSE, RAVLT, Babcock story recall Test, Corsi span, Digit span, TMT A-B, Stroop, Digit symbol test, figure copying. Clock drawing test, categorical, and phonological verbal fluency tests	Executive function, processing speed/ attention, visuospatial ability, memory
van de Beek et al. (2020)	The Netherlands	Retrospective cohort study (N/A)	To investigate the clinical characteristics and the predictors for dementia onset in prodromal DLB and AD.	MCI-LB (73)	MCI-AD (124)	Cognitive complaint, preserved ADL, MMSE ≥25, $z \leq -2$ in one NP domain	Probable DLB (McKeith et al., 2017)	MMSE, Visual Association Test, verbal learning test, TMT A-B, Stroop 1-3, Digit span, Letter fluency, Frontal Assessment Battery, VOSP number-location, dot counting, fragmented letters, Visual Association Naming Test	Executive and visuospatial function
Unger et al. (2019)	USA	Retrospective cohort study (2016–2018)	To characterize the diagnostic value of CVI testing, by comparing the CVI of patients with DLB, AD and pro-LBD.	pro-LBD (25)	DLB (62)	N/A	RBD and/or parkinsonism. No prior diagnosis PD or Parkinson clinical syndromes	MoCA	Visuospatial/ executive, attention, memory deterioration
Yoo et al., 2020	South Korea	Cross-sectional study (2015–2018)	To evaluate the striatal dopaminergic depletion and cerebral beta-amyloid deposition, and their association to cognitive function in LBD patients.	LBD-MCI (18)	HC (15)	N/A	Probable DLB (McKeith et al., 2017) and level-II PD-MCI (Litvan et al., 2012)	MMSE, Digit span, BNT, RCFT, SVLT, semantic and phonemic COWAT, Stroop	Attention, memory, executive function

MCI-P, patients where MCI was preceding Parkinson's disease; HC, healthy controls; PD-MOT, cognitively normal PD patients; NP, neuropsychological; DLB, dementia with lewy bodies; MCI-LB, MCI due to lewy body dementia; MCI-AD, dementia due to Alzheimer's disease; ADL, activities of daily living; IADL, instrumental ADL; Pro-LBD, prodromal lewy body disease; RBD, rapid eye movement (REM) sleep behavior disorder; CVI, color-vision impairment; MMSE, mini mental state evaluation; RAVLT, rey auditory verbal learning test; TMT, trail making test; VOSP, visual object and space perception battery; MoCA, montreal cognitive assessment; BNT, Boston naming test; RCFT, rey complex figure test; SVLT, Seoul verbal learning test; COWAT, controlled oral word association.

The Clock Drawing test and the TMT-B to be the most impaired cognitive tests in DLB-MCI. Compared to cognitively normal PD patients, the DLB-MCI patients had significantly reduced scores on tests measuring executive, attentional, visuospatial, and memory function. They found one test of visuospatial working memory and one of semantic verbal fluency to be able to fully separate those who develop LBD dementia from those who do not. The authors concluded that a worse visuospatial working memory and semantic verbal fluency is among factors putting a patient at higher risk of LBD dementia development.

Combining this with the neuroimaging findings, the authors conclude that patients with cognitive complaints and PD is best characterized as prodromal LBD (Donaghy and McKeith, 2014; McKeith et al., 2016) at first presentation, due to the extensive pathophysiological overlap.

The last three included studies did not, for various reasons, meet our full inclusion criteria. van de Beek et al. (2020) did not include HCs, Unger et al. (2019) lacked both HCs and had an unclear MCI definition, and lastly Yoo et al. (2020) both had an unclear MCI definition and a combined PD/DLB-MCI group.

Despite this, summed findings do point to DLB-MCI patients having poorer executive and visuospatial function compared to AD-MCI patients (van de Beek et al., 2020) and poorer executive and attentional functioning compared to HCs (Yoo et al., 2020). Moreover, a deterioration in attentional (Unger et al., 2019; van de Beek et al., 2020) and visuospatial/executive function (Unger et al., 2019) has been reported for DLB-MCI patients converting to dementia. Interestingly, van de Beek et al. (2020) also found that a poorer attentional function at first visit was associated with faster progression to dementia. The authors then concluded that this probably is an effect of these patients being closer to dementia, as DLB is characterized with more prominent attentional dysfunction early on in the disease course. Hence, the results point to a pattern of executive, visuospatial, and attentional deficits for persons living with DLB-MCI. Even so, the few existing studies provide rather heterogeneous findings, which is in line with Ciafone et al. (2020)'s findings.

When it comes to memory, the results are somewhat more mixed. While some of the studies find memory to be spared compared to AD-MCI (van de Beek et al., 2020), others find memory to be affected in DLB-MCI compared to HCs (Yoo et al., 2020). Lastly, both van de Beek et al. (2020) and Unger et al. (2019) report memory deterioration to be associated with conversion to dementia.

DISCUSSION

Despite being already 2 years since Donaghy et al. (2018) proposed criteria for DLB-MCI, only one study met the full inclusion criteria for this review (Massa et al., 2019). Further, only one study focused primarily on cognition (van de Beek et al., 2020), while the others had either color vision impairments or neuroimaging as their main focus. This shortage clearly underlines the paucity of research into DLB-MCI and emphasizes the need for well-controlled studies. Thus, the situation seems to be quite similar to that of January 2019 (Ciafone et al., 2020), and the cognition of DLB-MCI is still a scarce field of research. However, there are well-designed ongoing studies, that is, the Dementia Disease Initiation (DDI)-study (Fladby et al., 2017), that focuses on prodromal dementia symptoms, including prodromal DLB symptoms.

Because the main part of the included studies did not meet our full inclusion criteria, it is timely to question their utility for the scope of this review. Either way, the findings do however push toward a neuropsychological profile characterized by visuospatial, executive, and attentional deficits. In fact, this is in line with what we also found in mild DLB (Brønnick et al., 2016).

Furthermore, Massa et al. (2019) also found a worse visuospatial working memory and semantic verbal fluency to be a risk factor for LBD dementia development. One might question the specificity of this finding because it is in line with what is also found to be the case for conversion from MCI to AD (Belleville et al., 2017). Moreover, the results are inconclusive when it comes to the memory function of DLB-MCI patients. One might theorize that the large heterogeneity is due to some patients having a mixed DLB-AD pathology. However, caregivers to patients with DLB report memory impairment to be the most common symptom for both DLB and AD (Auning et al., 2011).

In addition, the substantial heterogeneity in the reported findings may firstly be due to the considerable variation in applied methods and definitions. One of the included studies used domain specific screening tools (Unger et al., 2019), while the others used more exhaustive neuropsychological tests. Further, some used composite scores (van de Beek et al., 2020; Yoo et al., 2020), while other analyzed isolated test scores (Massa et al., 2019; Unger et al., 2019). Moreover, some of the studies used standardized scores in the analyses (van de Beek et al., 2020; Yoo et al., 2020), while others used raw scores (Massa et al., 2019; Unger et al., 2019). This is somewhat problematic, due to age, sex, and educational differences between the subjects, as well as substantial differences in the normative basis for the different tests.

Second, we do not know whether all of the patients diagnosed as prodromal DLB did actually develop DLB. Only two of the included studies were longitudinal (Massa et al., 2019; van de Beek et al., 2020), and in these studies just about half of the subjects diagnosed as DLB-MCI actually developed dementia. In addition, in van de Beek et al. (2020), patients who received a probable DLB diagnosis at some point during follow-up were retrospectively defined as DLB-MCI (44%). Hence, these subjects probably did not reach the same criteria for a clinical diagnosis DLB-MCI at first presentation as the other ones, and grouping these together is thus problematic with respect to operationalization. The ideal situation would be to have a group of patients clinically diagnosed with DLB-MCI who later on received a definite DLB diagnosis.

Third, the heterogeneity of the findings may also be a natural cause of the varying terms and diagnostic criteria used in the different studies. Indeed, only van de Beek et al. (2020) stated a clear MCI definition together with McKeith et al. (2017) criteria

for probable DLB. The other studies chunked PDD and DLB together to an LBD-MCI group, or had a restricted focus on parkinsonism or RBD, not taking the other DLB core criteria into account. For example, Massa et al. (2019) did not use the 1-year rule as is stated for research purposes to differentiate between PDD and DLB. Furthermore, Yoo et al. (2020) also chunked PDD and DLB together in an LBD-MCI group, of which only four of them were diagnosed as DLB-MCI, as opposed to 14 as PD-MCI. This bias clearly affects the utility of this study as a DLB-MCI study; in fact, it may be more representable as a PD-MCI study.

Future studies must therefore apply the newly published consensus criteria (McKeith et al., 2020) to make comparison between studies possible. Here MCI is defined as a cognitive concern proposed by the patient, an informant, or a clinician, in addition to objective impairment in one or more cognitive domains and preserved ADL. In order to receive a diagnosis of DLB-MCI, the patients must also meet the criteria for probable or possible DLB (McKeith et al., 2017, 2020). In the time ahead, we accordingly expect a substantial amount of studies using these new criteria. Despite the budding amount of research suggesting visuospatial, attentional, and executive deficits in DLB-MCI, a sound conclusion regarding the cognitive profile of DLB-MCI awaits a future body of research using the McKeith et al. (2020) criteria. Additionally, studies with higher focus on new potential biomarkers and a larger number of included subjects is important to further subgroup this heterogeneous group and to get more robust and representative findings.

Lastly, some of the included studies proposed that a DLB-MCI diagnosis might not be fruitful, due to a both substantial symptomatic heterogeneity and overlap (Massa et al., 2019; van de Beek et al., 2020). At this point, however, it seems like a rather premature proposition due to the lack of well-controlled studies. As mentioned, Massa et al. (2019) suggests a prodromal LBD diagnosis for patients with both PD and cognitive complaints, due to their finding of a substantial pathophysiological overlap between PD-MCI and DLB-MCI patients. This may, however, be a spurious effect because they did not adhere to the 1-year rule and chunked prodromal PDD and DLB patients together in their prodromal LBD group. Hence, a pathophysiological overlap is as expected. Nonetheless, this discourse points to the necessity of conducting proper neuropsychological assessments of all patients with PD.

Study Limitations and Strengths

The strength of this study is the thorough and systematic search in which it is based on, thus making it unlikely that we missed relevant studies. Our study does, however, have some limitations, with the most obvious being the few studies included. All of the studies also had few study subjects included. One can therefore not use the findings from this study to validate the neuropsychological profile of DLB-MCI. Another minor limitation is that we are building on a recently published review without knowing the exact search strategy of this study. Moreover, our searches were not performed in the exact same databases as Ciafone et al. (2020).

CONCLUSION

As Ciafone et al. (2020), our findings indicate that DLB-MCI patients have a pattern of executive, visuospatial, and attentional deficits. Hence, it resembles that of DLB. However, there is a clear lack of well-controlled studies with a sizable number of study subjects focusing on cognition in DLB-MCI. One major reason for this might be the challenge of identifying these patients due to both heterogeneous symptom presentation in the prodromal stage and the lack of clear definition. With recently published research criteria for DLB-MCI (McKeith et al., 2020), it might be easier to identify these patients, and a wealth of studies is therefore expected to come in the near future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MH conceived the research question, which was then discussed with all the authors. MH performed the searches and drafted the manuscript. MB was the main supervisor. At inclusion ambiguous cases were discussed between MH and MB. LC and AR contributed to the hypothesis and revised the manuscript. At the end all authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.597579/full#supplementary-material

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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FTY720 Prevents Spatial Memory Impairment in a Rat Model of Chronic Cerebral Hypoperfusion via a SIRT3-Independent Pathway

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Vascular dementia (VD) and Alzheimer's disease (AD) are the most prevalent types of late-life dementia. Chronic cerebral hypoperfusion (CCH) contributes to both AD and VD. Recently, accumulating evidence has indicated that fingolimod (FTY720) is neuroprotective in acute cerebral ischemic stroke animal models, and the drug is now being used in clinical translation studies. However, fewer studies have addressed the role of FTY720 in chronic cerebral hypoperfusion (CCH)-related brain damage. In the present study, to investigate whether FTY720 can improve CCH-induced spatial memory loss and its underlying mechanism, two-vessel occlusion (2VO) rats were administered intraperitoneal FTY720 (1 mg/kg) for 7 consecutive weeks from post-operative day 8. Spatial memory was tested using the Morris Water Maze (MWM), and the rats' brains were harvested to allow molecular, biochemical, and pathological tests. We found that FTY720 treatment significantly reduced the escape latency and increased the target quadrant swimming time of the 2VO rats in the MWM task. The improvement in memory performance paralleled lower levels of pro-inflammatory cytokines and Iba-1 positive cells in the hippocampus of the 2VO rats, indicating that FTY720 had a beneficial effect in mitigating neuroinflammation. Furthermore, we found that FTY720 alleviated mitochondrial dysfunction in 2VO rats, as manifested by lower malondialdehyde levels, higher ATP content, and upregulation of ATP synthase activity in the hippocampus after treatment. FTY720 had no effect on the CCH-induced decrease in the activity of hippocampal Sirtuin-3, a master regulator of mitochondrial function and neuroinflammation. In summary, the results showed that FTY720 can improve CCH-induced spatial memory loss. The mechanism may involve Sirtuin-3-independent regulation of mitochondrial dysfunction and neuroinflammation in the hippocampus. The present study provides new clues to the pathological mechanism of CCH-induced cognitive impairment.

Keywords: chronic cerebral hypoperfusion (CCH), neuroinflammation, mitochondrial dysfunction, mitophagy, fingolimod

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INTRODUCTION

Chronic cerebral hypoperfusion (CCH) is an important pathophysiological process underlying Alzheimer's disease (AD) and vascular dementia (VD) (Duncombe et al., 2017). Animal models of CCH, including the two-vessel occlusion (2VO) rat model and the bilateral carotid artery stenosis (BCAS) mouse model, mimic the cognitive impairment of AD and VD patients (Venkat et al., 2015; Tuo et al., 2020; Yao et al., 2020). Mitochondrial dysfunction under CCH conditions can induce oxidative stress, as well as neural and synaptic damage, thus triggering microglial activation and astrogliosis (Du et al., 2017). Therefore, mitochondrial dysfunction is a key upstream event for other pathological changes in CCH. Despite this, the mitochondrial mechanism of CCH-induced cognitive impairment remains elusive.

Deacetylase sirtuin-3 (SIRT3) is a master regulator of mitochondrial function (Hirschey et al., 2010). By deacetylating and enhancing the activity of superoxide dismutase-2 (SOD2), isocitrate dehydrogenase-2 (IDH2), and multiple enzymes in the electron transmission chain, SIRT3 can prevent oxidative damage and promote mitochondrial bioenergetics (Yu et al., 2012; Gao et al., 2018). Chronic administration of SIRT3 agonist honokiol (HNK) prevents oxidative stress, neuroinflammation, and spatial memory impairment in the 2VO rat model (Guo et al., 2019). Thus, SIRT3 may be involved in CCH-related mitochondrial dysfunction.

Neurons recycle damaged mitochondria through mitophagy, thus maintaining cellular homeostasis and inhibiting mitochondria-dependent cell apoptosis (Lou et al., 2020). AD manifests mitophagy deficiency, resulting in impaired mitochondrial accumulation, neuronal apoptosis, and ultimately cognitive impairment (Fang et al., 2019). One interesting question is whether CCH can induce a similar pathological change, since it is reported that SIRT3 can induce mitophagy in mouse heart (Li et al., 2018).

Fingolimod (FTY720) was approved as the first oral drug for treating relapsing forms of multiple sclerosis in 2010 (Volpi et al., 2019). It subsequently proved to be a promising treatment for stroke (Wang et al., 2020). The drug binds to sphingosine 1-phosphate receptors (S1PRs) and can exert neuroprotection in multiple models of neurological disease. It can prevent memory loss in rodent models of AD (McManus et al., 2017) and autism (Wu et al., 2017), and it alleviates hypoxic-ischemia injury in the neonatal rat brain (Serdar et al., 2016). This suggests that FTY720 exerts its neuroprotective effects by mitigating oxidative stress and neuroinflammation, which also underlie CCH-related neuronal damage. One recent study further reported that the drug can prevent working memory deficits in a mouse model of BCAS by regulating microglia polarization in the corpus callosum (Qin et al., 2017). However, since BCAS mice show normal spatial memory function, it remains unclear whether FTY720 can prevent CCH-induced hippocampus-dependent spatial memory loss.

We sought to determine whether FTY720 administration during the chronic phase could prevent spatial impairment in the 2VO rat model and, if so, whether SIRT3 or other mitochondrial mechanisms are involved.

MATERIALS AND METHODS

Animals

Sixty six-week-old male Sprague–Dawley rats weighing 200 ± 20 g were raised in the Animal Experiment Center of Zhongnan Hospital, Wuhan University. They were kept at a temperature of 22 ± 1°C and in a controlled 12-h light/dark cycle. Food and water were available *ad libitum* throughout the study. The rats were randomly divided into four groups after 7 days of acclimation: sham with normal saline administration (sham+NS), sham with FTY720 treatment (sham+FTY720), 2VO with normal saline administration (2VO+NS), and 2VO with FTY720 treatment (2VO+FTY720). The timeframe of this study is presented in **Figure 1**. All experimental procedures were conducted with permission from the Ethics Committee of Animal Experimentation of Wuhan University and in strict accordance with the ARRIVE Guidelines (Kilkenny et al., 2010).

Two-Vessel Occlusion Surgery and Drug Administration

The 2VO procedure was performed as previously described (Farkas et al., 2004; Sanderson and Wider, 2013; Hu et al., 2019). After anesthesia induction by inhalation of 4% isoflurane for 3–5 min, rats were maintained under anesthesia using 2% isoflurane (0.5 L/min). A 2-cm incision was made in the middle of the cervical region, with the rat in a supine position. Next, the muscle and fascia were dissected bluntly. The carotid artery was separated and exposed within the carotid sheath. A 4–0 silk suture was used to permanently ligate the carotid. After 30 min, the other carotid was ligated in the same way. Sham groups were subjected to the same procedure without carotid ligation. Rats were placed in a sterilized blanket during the surgery to keep their bodies warm. After skin suturing, the rats were put back into their cages with free access to food and water. FTY720 (F126599; Aladdin) was dissolved in 0.9% normal saline to prepare 10% solutions. After 8 days of surgery, the rats received FTY720 (1 mg/kg) or normal saline treatment by intraperitoneal injection once a day for 7 consecutive weeks. After that, the Morris water maze (MWM) test was carried out.

Morris Water Maze

The MWM was performed as previously described (Vorhees and Williams, 2006). The round swimming arena was divided into four quadrants with identical areas. A platform was placed 1 cm below the water in the middle of the target quadrant. The rats were gently placed in the water, and the hidden platform was the only escape. The time each rat spent finding the platform was recorded as the escape latency, and swimming speed was also recorded to evaluate locomotive ability. The escape latency of rats that failed to find the platform within 1 min was recorded as 60 s. Whether or not the platform was found, each rat was allowed to stand on the platform for 15 s after the test. During the 5 days of the training phase, the rats were released to swim from four different locations on opposite sides of the platform in sequence. On the 6th day for the probe trial, the platform was removed and rats started from the opposite position where the platform was located. The time spent in the target quadrant was recorded.

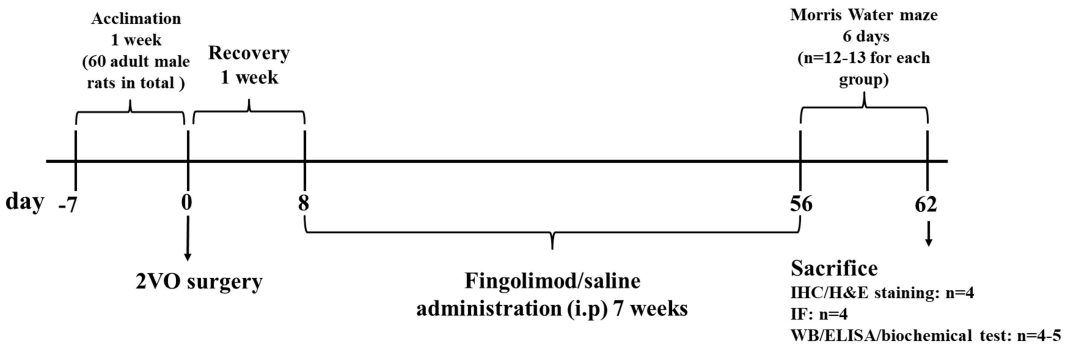

FIGURE 1 | Schematic representation of the experimental design. 2VO, two-vessel occlusion; IHC, immunohistochemistry; H&E staining, hematoxylin and eosin staining; IF, immunofluorescence; WB, western blot; ELISA, Enzyme-linked immunosorbent assay.

Western Blot

Rats were sacrificed for immunoblotting after the behavioral test. The brain was collected on ice to prepare for hippocampal dissection. Tissues of the hippocampus were immediately frozen in liquid nitrogen and stored at −80°C. RIPA (P0013B; Beyotime Biotechnology, Shanghai, China) buffer with PMSF (ST505; Beyotime Biotechnology, Shanghai, China) was used to lyse the tissues. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was transferred into a new EP tube and prepared for protein concentration using a BCA Protein Assay Kit (P0012S; Beyotime Biotechnology, Shanghai, China). Extracted proteins (20 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a 0.45-μm pore size polyvinylidene fluoride membrane (IPVH00010; Millipore, MA, USA). Next, 5% (weight/volume) skim milk power was added to TBS/0.1% Tween-20 (0.1% TBST) to block non-specific protein-binding on the membrane. Primary antibody was diluted in 0.1% TBST and incubated with the membrane overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody (1:10,000; SA00001-2; Proteintech, Wuhan, China) diluted in 0.1% TBST was used to incubate the membranes at room temperature for 1 h. An enhanced chemiluminescence system (Tanon-5200, Shanghai, China) was used for visualization and semi-quantitation of target protein expression. The following antibodies were used in our research: anti-postsynaptic density-95 (anti-PSD95; 1:1,000; ab18258; Abcam, Cambridge, UK), anti-SIRT3 (1:1,000; 2627s; Cell Signaling Technology, MA, USA), anti-SOD2 (1:1,000; ab68155; Abcam, Cambridge, UK), anti-IKBα (1:2,000, ab32518 Abcam, Cambridge, UK), anti-SOD2 acetyl K68 (1:1,000; ab137037; Abcam, Cambridge, UK), anti-p62 (1:10,000; ab56416; Abcam, Cambridge, UK), anti-cyclooxygenase-4 (COX4; 1:1,000; PA5-29992, Thermo, MA, USA), and anti-GAPDH (1:10,000; Proteintech, Wuhan, China).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Hu et al., 2019). Anesthetized rats were perfused from the heart with 150 mL 0.9% saline and then 150 mL 4% paraformaldehyde. Brains were dissected, dehydrated, and embedded in paraffin. Paraffin-embedded brains were sectioned coronally into 10-μm thick slices. Antigen retrieval was performed by heating the sections in citrate buffer after deparaffinization and rehydration. Slices were then blocked with 10% goat serum TBS and incubated with primary antibody-ionized calcium-binding adapter molecule 1 (anti-Iba-1; 1:400; 10904-1-AP, Proteintech) overnight at 4°C. Biotinylated secondary antibody was applied for 30 min at room temperature. After that, avidin–biotin complex followed by 3,3′-diaminobenzidine solution (P0202; Beyotime Technology) were applied to the tissues for 30 and 10 min, respectively. Finally, the slices were counterstained using hematoxylin for nuclear staining.

Hematoxylin and Eosin Staining and Cell Counting

Slices for cell numbers in the cornu ammonis 1 (CA1) region were subjected to hematoxylin and eosin (H&E) staining. The hematoxylin stained the nuclear chromatin, while the eosin stained the cytoplasm. All samples were observed under an Olympus BX53 microscope at 400x magnification. The same CA1 region of the images was captured from three slices in each animal using image analysis software (Olympus Stream). The number of cells and Iba-1-positive cells per mm^2 in the CA1 region and dentate gyrus (DG) region were calculated using ImageJ software (NIH, USA).

Immunofluorescence

Immunofluorescence was performed as previously described (Kaiser and Feng, 2019). Anesthetized rats were perfused from the heart with 0.9% saline (150 mL) and then 4% paraformaldehyde. Brains were dissected and submerged in 4% paraformaldehyde overnight at 4°C followed by 30% sucrose/PBS dehydration. The brains were then covered in optimal cutting temperature compound and stored at −80°C to await sectioning into 10 μm-thick slices. Subsequently, the slices were blocked using 10% goat serum TBS and incubated with primary antibodies overnight at 4°C. For mitophagy event detection, fluorescent secondary antibodies from different

hosts were applied for 30 min at room temperature. DAPI was applied to mark the nuclei after the secondary antibody was washed off. The primary antibodies used were as follows: anti-LC3 (1:200; ab48394, Abcam), anti-translocase of the outer mitochondrial membrane member-20 (anti-TOMM20; 1:100; ab56783, Abcam). Alexa Fluor 594-conjugated secondary antibody (1:200; ab150080; Abcam) and Alexa Fluor 488-conjugated (cross-adsorbed) secondary antibody (1:100; A11029, Invitrogen) were applied. Anti-PSD95 (1:200; ab18258; Abcam, Cambridge, UK) primary antibody and Alexa Fluor 594-conjugated secondary antibody (1:200; ab150080; Abcam) were applied for synaptic staining. All samples were observed under an Olympus BX53 microscope at 400x magnification. The same CA1 region of the images was captured from three slices in each animal using image analysis software (Olympus Stream). Area Fraction of PSD95 and colocation of LC3 and TOMM20 was identified using Image J software (NIH, USA).

Enzyme-Linked Immunosorbent Assay

Levels of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the rat hippocampus were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions, with the optical density value measured using a microplate reader (DR-200Bs; DiaTek Company, Wuxi, China) at a wavelength of 450 nm. The following ELISA kits were used: Rat TNF-α ELISA Kit (ELK1396; ELK Biotechnology, Wuhan, China), Rat IL-1β ELISA Kit (ELK1272 ELK Biotechnology, Wuhan, China), and Rat IL-6 ELISA Kit (ELK5684; ELK Biotechnology, Wuhan, China).

Biochemical Detection

The levels of ATP, SOD2, and ATP synthase activity in the hippocampal homogenate of rats were detected using colorimetric assay kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China), according to the manufacturer's instructions. A spectrophotometer was used in the test process. The following assay kits were used: SOD2 activity (A001-2), ATP content (A095-1-1), Mitochondrial extraction (G006-1-1), and ATP synthase activity (A089-5-1).

Statistical Analysis

Data are presented as mean ± standard error of the mean. The Shapiro-Wilk test did not show a significant departure from normality in the distribution of the parameters. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, USA). In western blot, immunohistochemistry, ELISA, biochemical analysis, and part of the MWM test (time spent in target quadrant of probe trial; swimming speed), differences between groups were determined using one-way analysis of variance (ANOVA) followed by the *post-hoc* Bonferroni test. Differences in escape latency during the five training periods were analyzed using two-way ANOVA, and $p < 0.05$ were considered statistically significant.

RESULTS

FTY720 Attenuated Spatial Memory Impairment Induced by CCH in 2VO Rats

The MWM was applied to test the spatial memory of rats in the four groups. **Figure 2A** shows that, over the 5 consecutive days of the training phase, there was no significant difference in escape latency in the first 3 days, while 2VO-NS rats spent more time finding the platform than the 2VO-FTY720 rats on the 4th and 5th days ($p < 0.05$). On the probe trial phase, 2VO-FTY720 rats exhibited a higher percentage of time in the target quadrant than 2VO-NS rats ($p < 0.05$; **Figure 2C**). There was no significant difference in swimming speed among the four groups ($p > 0.05$; **Figure 2B**). Representative swimming paths were shown in **Figure 2D**. These results suggested that FTY720 improved spatial memory in 2VO rats without affecting their locomotive ability.

FTY720 Did Not Affect Hippocampus CA1 Neuron Loss in 2VO Rats

H&E staining of the hippocampus CA1 region showed that the cell number was lower in 2VO-NS rats than in sham-NS rats ($p < 0.05$; **Figures 3A,B**). Compared with 2VO-NS rats, 2VO-FTY720 rats had a tendency toward fewer hippocampal CA1 neurons, although the difference was not significant ($p > 0.05$; **Figures 3A,B**). To further study whether FTY720 influenced CCH-induced synaptic damage, we tested PSD95 expression in the hippocampus by western blotting. As shown in **Figure 3C**, PSD95 expression was lower in 2VO-NS rats than in sham-NS rats ($p < 0.05$), while FTY720 administration conferred higher PSD95 expression in 2VO rats ($p < 0.05$). Immunofluorescence was conducted to further confirm PSD95 expression. In agreement with the western blot results, FTY720 intervention increased the hippocampal PSD95 immunoactive area in 2VO rats ($p < 0.05$, **Figures 3D,E**).

FTY720 Alleviated CCH-Induced Neuroinflammation in the Hippocampus of 2VO Rats

Iba-1 is a marker of microglia, so we counted Iba-1-positive cells in the hippocampal CA1 and DG regions. The 2VO-NS rats expressed more Iba-1-positive cells than the Sham-NS rats in the hippocampus CA1 and DG regions ($p < 0.05$; **Figures 4A,B**). FTY720 significantly reduced the number of Iba-1-positive cells in both hippocampus subregions in 2VO rats ($p < 0.05$; **Figures 4A,B**). We then tested the levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, using ELISA. The 2VO-NS rats showed higher expression of these pro-inflammatory factors in the hippocampus ($p < 0.05$; **Figure 4C**), and FTY720 treatment reduced TNF-α, IL-1β, and IL-6 release in the hippocampus of 2VO rats ($p < 0.05$; **Figure 4C**). Since IKBα inhibits the release of downstream inflammatory cytokines (Oeckinghaus et al., 2011), we also tested the expression of IKBα and found that 2VO-NS rats showed lower IKBα expression in the hippocampus, while FTY720 treatment upregulated IKBα levels ($p < 0.05$; **Figure 4D**).

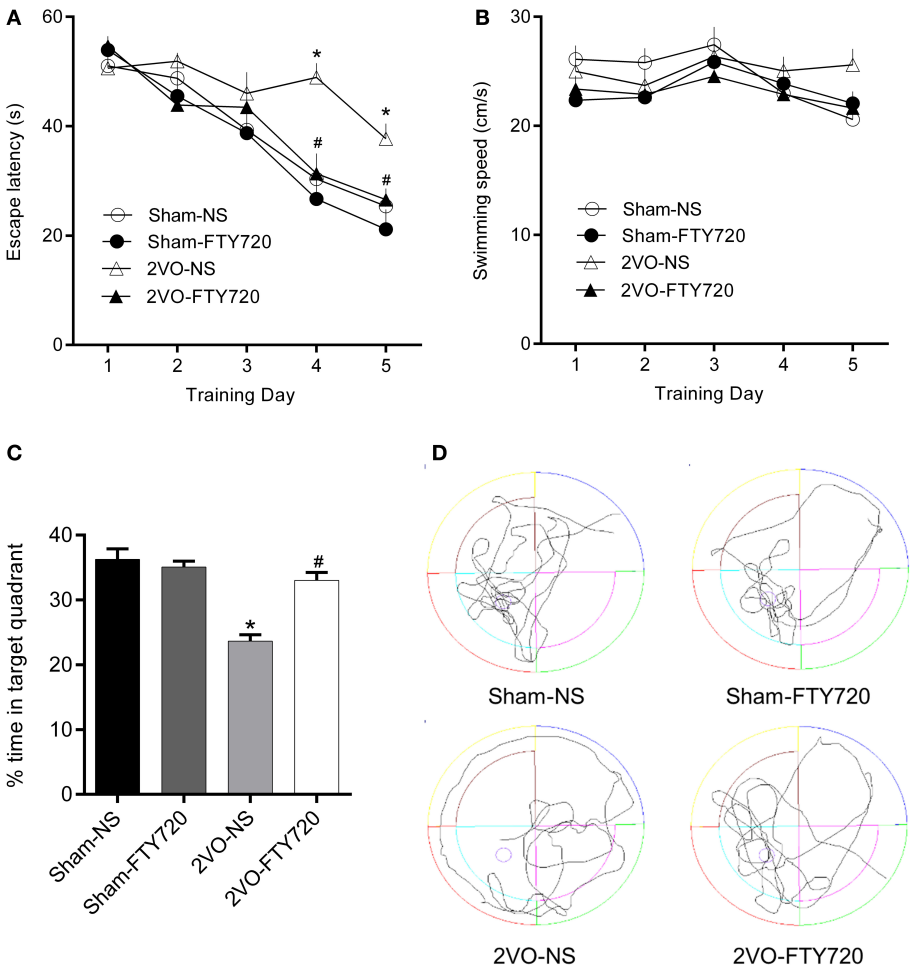

FIGURE 2 | The escape latency **(A)**, swimming speed during the training phase, **(B)** percentage of time spent in target quadrant **(C)**, and representative swimming paths **(D)** in the probe trial of MWM task of FTY720 or Saline treated rats after the 2VO surgery. All data are shown as mean ± S.E.M, $n = 12$–13 rats for each group. *compared to sham-NS rats, $p < 0.05$, #compared to 2VO-NS rats, $p < 0.05$.

Overall, these data suggested that FTY720 decreased microglia activation and regulated the concentration of inflammatory factors in response to CCH.

FTY720 Improved Mitochondrial Dysfunction in the Hippocampus of 2VO Rats

Oxidative stress originating from mitochondrial dysfunction is considered a crucial pathological process in CCH (Du et al., 2013; Ham and Raju, 2017). To further explore whether FTY720 could alleviate hippocampal mitochondrial dysfunction in 2VO rats, we tested malondialdehyde (MDA) and ATP content, as well as ATP synthase activity in the hippocampus of all groups of rats. The 2VO-NS rats showed increased MDA levels ($p < 0.05$; **Figure 5A**), as well as decreased ATP content and ATP synthase activity compared to Sham-NS and Sham-FTY720 rats ($p < 0.05$; **Figures 5B,C**). Meanwhile, FTY720 decreased the MDA levels, but increased the ATP content and ATP synthase activity in the 2VO rat hippocampus ($p < 0.05$; **Figures 5A–C**). These results indicated that FTY720 could ameliorate the hippocampal mitochondrial dysfunction induced by CCH.

FTY720 Had No Effect on SIRT3 Activity in the Hippocampus of 2VO Rats

There was no significant difference in SIRT3 protein expression among the four groups after FTY720 treatment ($p > 0.05$; **Figure 6A**). SOD2 is the downstream target molecule of SIRT3. We found that 2VO-NS and 2VO-FTY720 rats showed higher acetylated-SOD2 K68 levels than controls in the hippocampus ($p < 0.05$; **Figure 6B**). However, FTY720 treatment did not improve acetylated-SOD2 K68 levels in 2VO rats ($p < 0.05$; **Figure 6B**). Consistent with the SOD2 acetylation level, FTY720 did

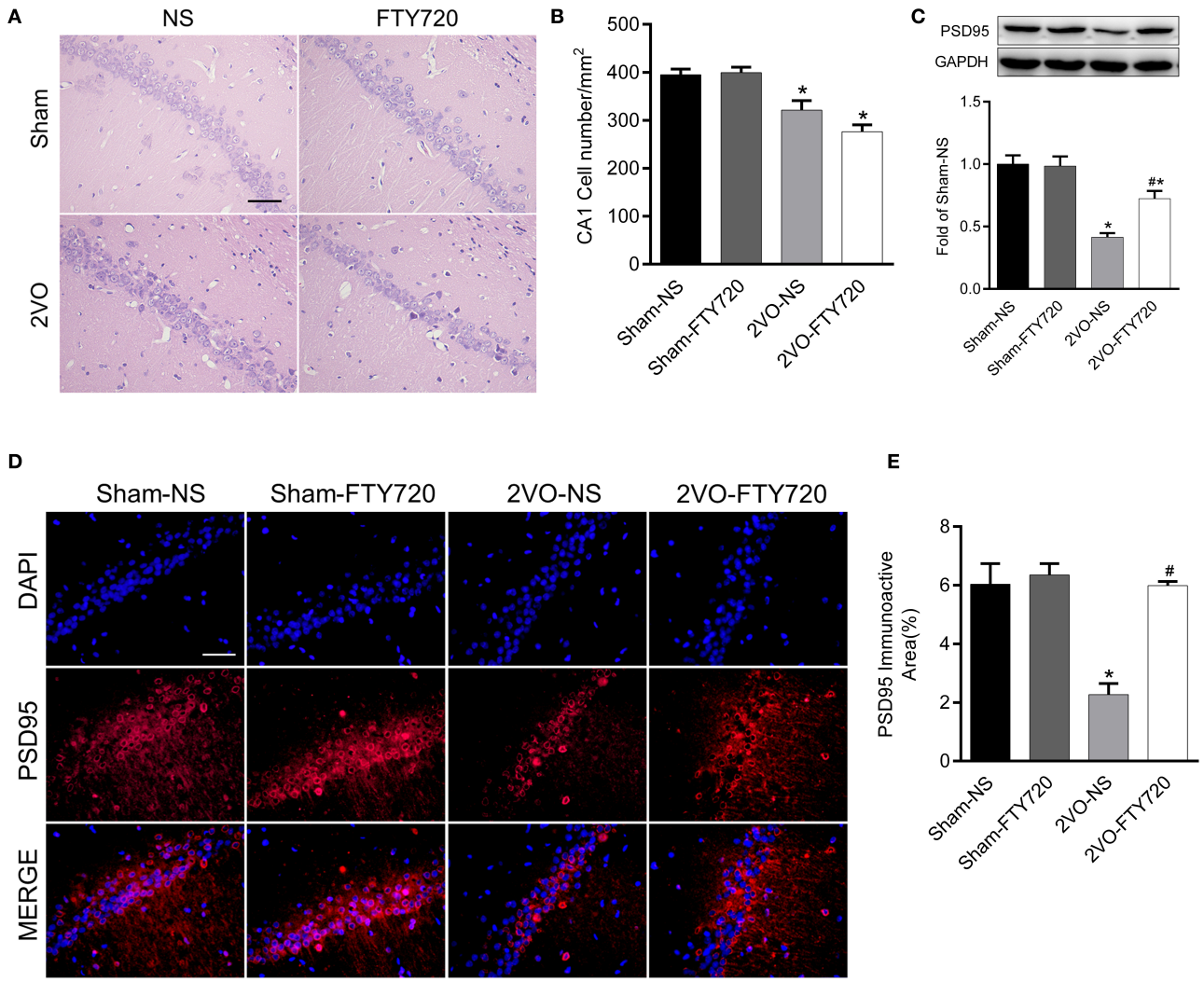

FIGURE 3 | The effect of FTY720 on CA1 cell number and PSD95 expression. **(A)** Representative H&E staining image of rat hippocampus CA1 region (Scale bar = 50 μm, 400×). **(B)** Statistical bar graph of cell numbers in hippocampus CA1 region. **(C)** Representative western blot bands of rat hippocampal PSD95 and quantitative analysis. Data was normalized to GAPDH and expressed as fold of sham-NS **(D,E)** Representative image and statistical analysis of PSD95 immunofluorescence staining (Scale bar = 50 μm, 400×). Data were shown as mean ± S.E.M. $n = 4$ rats for each group. $^{*}p < 0.05$ compared with the sham-NS group. $^{\#}p < 0.05$ compared with the 2VO-NS group.

not increase SOD2 activity in the hippocampus of 2VO rats (**Figure 6C**).

FTY720 Did Not Influence Mitophagy in the Hippocampus CA1 Region of 2VO Rats

The autophagy markers LC3 and mitochondria marker TOMM20 were stained in the CA1 region (**Figure 7A**). TOMM20 overlapping with LC3 ratio was identified as the mitophagy event. However, we found no difference in the overlapping rate of TOMM20 and LC3 among the four groups ($p > 0.05$; **Figure 7B**), and there was no significant difference among the four groups in the autophagic flux marker p62 ($p > 0.05$; **Figure 7C**).

DISCUSSION

The present study proved that FTY720 can prevent CCH-induced spatial memory impairment and mitochondrial dysfunction, without affecting SIRT3 activity.

Bilateral common carotid artery occlusion results in blood flow redistribution in the Willis arterial circle in rats (Farkas et al., 2007). The chronic brain ischemia phase ranges from 4 days to 8–12 weeks after the surgery. The mortality rate reported in previous studies of 2VO surgery has varied from 26 to 50%, and it stops increasing after 1 week (Wang et al., 2014; Li et al., 2015). Therefore, we chose to start drug administration on the 8th day after surgery to avoid any acute ischemia effect.

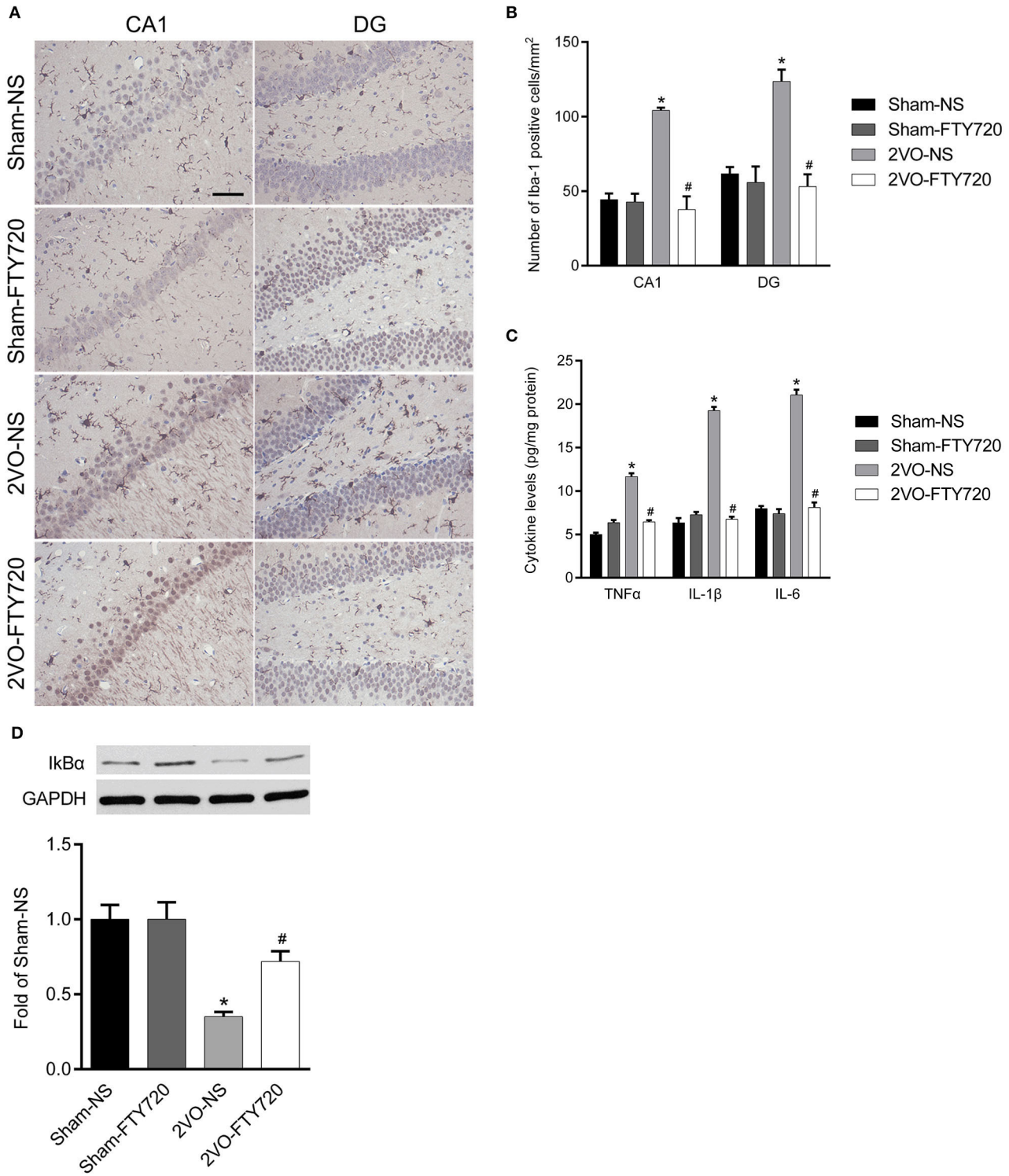

FIGURE 4 | The modulation of FTY720 to CCH-induced hippocampal neuroinflammatory response. **(A)** Representative immunohistochemistry images of Iba-1 positive cells in the hippocampus of rats (Scale bar = 50 μm, 400×). **(B)** Quantitation of Iba-1 positive cells in the hippocampus CA1 and DG regions with bar graph. **(C)** Quantitative bar graph of pro-inflammation cytokines expression in the hippocampus. **(D)** Representative IkBα western blot bands in the hippocampus of animals. Protein level was normalized to the loading of GAPDH and expressed as fold of sham-NS. Data were shown as mean ± S.E.M. $n = 4–5$ rats for each group. $^{*}p < 0.05$ compared with the sham-NS group. $^{\#}p < 0.05$ compared with the 2VO-NS group.

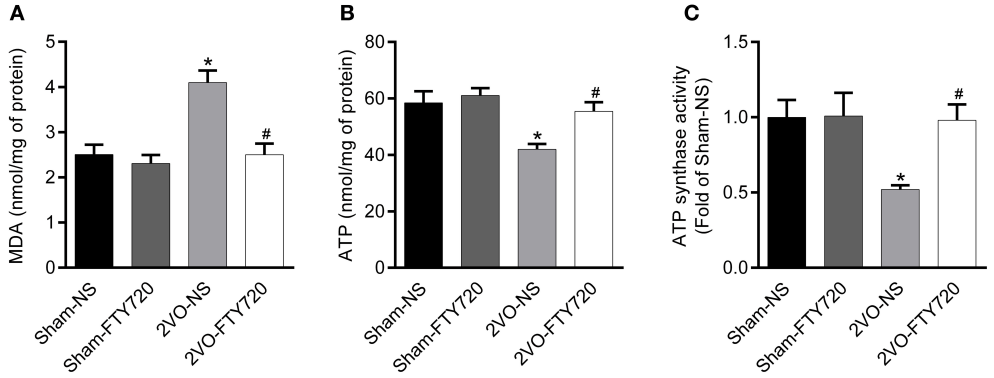

FIGURE 5 | Quantitation of MDA **(A)**, ATP content **(B)** as well as ATP synthase activity **(C)** in the hippocampus of FTY720 or saline treated rats after the 2VO surgery. Data are shown as mean ± S.E.M. $n = 4$–5 rats for each group. $^{*}p < 0.05$ compared with the sham-NS group. $^{\#}p < 0.05$ compared with the 2VO-NS group.

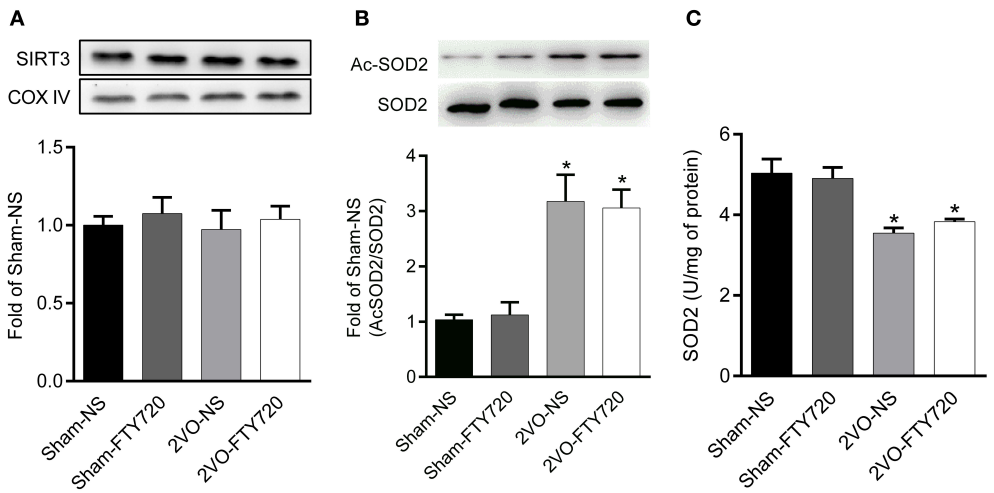

FIGURE 6 | Representative SIRT3 **(A)**, acetylated SOD2 K68 (Ac-SOD2 K68) **(B)** expressions, SOD2 activity **(C)** in the hippocampus of rats in each group. SIRT3 and SOD2 K68 expression in the hippocampus were normalized to loading of COX IV and SOD2, respectively, and were expressed as fold of sham-NS. Data were shown as mean ± S.E.M. $n = 4$–5 rats for each group. $^{*}p < 0.05$ compared with the sham-NS group.

During the chronic cerebral ischemia phase, neuron death and synapse loss mirror cognitive impairment was induced by CCH (Du et al., 2017). In the present study, FTY720 had no effect on neuron numbers in the hippocampal CA1 region after 2VO, while it has protected against neuronal loss in other studies (Asle-Rousta et al., 2013; Wu et al., 2017). This discrepancy may have arisen because FTY720 intervention was started at different times. Cechetti et al. (2012) calculated neuron numbers in the hippocampal CA1 region at multiple time points, finding that the number of NeuN-positive cells was markedly lower than in sham-operated rats 1 week after surgery. However, there was no significant difference in NeuN-labeled cell number between 1 week and 3 months after surgery (Cechetti et al., 2012), suggesting that no significant neuronal apoptosis occurs during this chronic cerebral ischemia phase. In the present study, we chose to use FTY720 8 days after surgery, so the drug could not prevent early neuron loss. In other neurological disease models in which neuronal loss has been ameliorated by FTY720, preconditioning or early drug administration before neural damage have been applied (Asle-Rousta et al., 2013; Ren et al., 2017).

Neuronal loss normally parallels a decrease in synaptic density, and synaptic function is of vital importance for cognition (Li et al., 2019; Wang et al., 2019; Che et al., 2020). CCH results in a low oxygen supply to the electron transport chain, generating excessive reactive oxygen species (ROS), inducing oxidative stress, and thus facilitating neuroinflammation in the hippocampus (Farkas et al., 2007). According to previous studies (Rao et al., 2012; Bollinger et al., 2019), oxidative stress and neuroinflammation cause synaptic damage. FTY720 has been reported to mitigate oxidative stress and neuroinflammatory reactions in animal models of neurological diseases (Colombo et al., 2014; Serdar et al., 2016). Corroborating this, our results showed that FTY720 alleviated oxidative stress

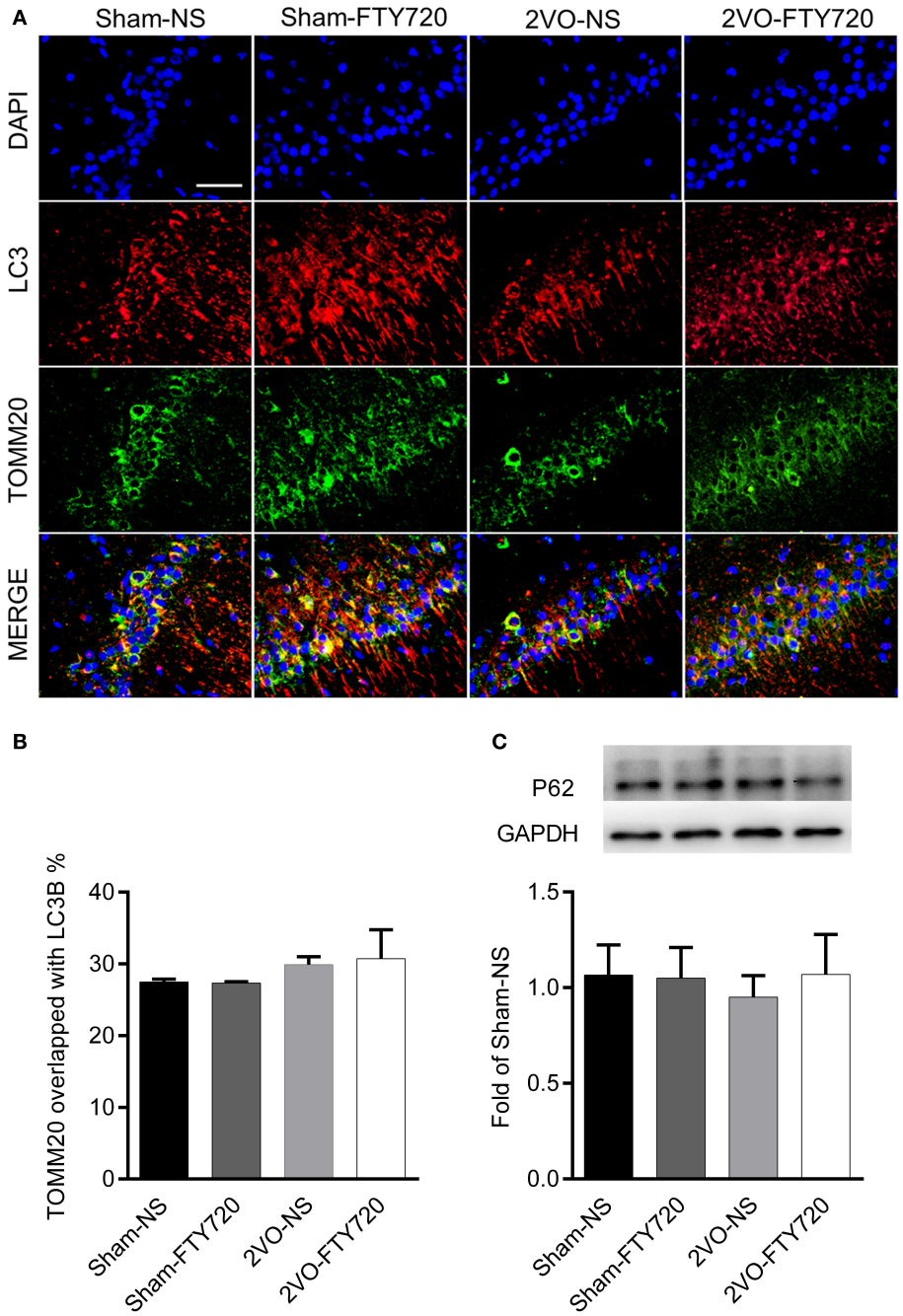

FIGURE 7 | The effect of FTY720 on hippocampal mitophagy markers of 2VO rats. **(A)** Representative immunofluorescent images of LC3 and TOMM20 colocalization in the hippocampus CA1 region. **(B)** The effect of FTY720 on hippocampal mitophagy markers of 2VO rats. **(C)** Representative hippocampal p62 immunoblot bands of FTY720 or saline treated rats after the 2VO surgery. p62 levels were normalized to GAPDH and expressed as fold of sham-NS in the lower panel. All data were shown as mean ± S.E.M. $n = 4$–5 rats for each group. Scale bar=50 μm, 400×.

and neuroinflammation in the hippocampal CA1 region of 2VO rats, and that it increased PSD95 and recovery of memory deficits. It follows that mitigated oxidative stress and neuroinflammation, as well as subsequent improved synaptic function, underlies the cognitive protective effect of FTY720.

Mitophagy is a key process to guarantee normal mitochondrial function by alleviating oxidative stress (Baechler et al., 2019; Zhang et al., 2019). Recently, mitochondrial damage due to mitophagy reduction has been revealed as an important factor in AD-related cognitive impairment (Fang et al., 2019). In the present study, there was no change in colocalization of the

autophagy marker LC3 and the mitochondrial membrane protein TOMM20 after FTY720 administration, indicating that FTY720 has no impact on mitophagy. Expression of p62 remained unchanged after FTY720 intervention, indicating that FTY720 does not affect autophagic flux, since p62 is important in the transfer of ubiquitinated substrates to autophagosomes (Wen et al., 2019). Therefore, mitophagy may not be the main mechanism responsible for mitochondrial dysfunction in CCH, as it is in AD.

The mitochondrial deacetylase SIRT3 is the master regulator of mitochondrial function (Baeza et al., 2016; Salvatori et al., 2017; Gao et al., 2018). SIRT3 deficiency aggravates cerebral ischemia-induced oxidative stress and neuroinflammation (Yang et al., 2020). Chronic administration of SIRT3 agonist honokiol (HNK) also prevents oxidative stress, neuroinflammation, and spatial memory impairment in 2VO rats (Guo et al., 2019). As the downstream molecule, SOD2 is acetylated by SIRT3, so its K68 acetylation level can reflect SIRT3 activity (Chen et al., 2011). Our research found that SOD2 K68 acetylation level, rather than SIRT3 protein level, decreased in the CCH rat hippocampus. This result indicates that SIRT3 post-translational change may be involved in CCH-induced mitochondrial dysfunction. Since SIRT3 can be post-translationally modified through phosphorylation or S-sulfhydration (Liu et al., 2015; Yuan et al., 2019), further study is needed to ascertain how CCH-induced post-translational modification of SIRT3 is related to cognitive impairment. FTY720 does not influence SIRT3 protein expression or its activity in the hippocampus of 2VO rats, indicating that chronic FTY720 administration involves an alternative pathway.

One limitation of the present study was the lack of further study into the mechanism by which FTY720 ameliorates CCH-induced mitochondrial dysfunction. A recent *in vitro* study found that FTY720 can increase neuronal mitochondrial stability and restore mitochondrial dynamics under conditions of oxidative stress by modulating S1PRs (Martin-Montanez et al., 2019). Further studies are needed to clarify how membrane-anchored S1PRs can couple with mitochondria when *in vivo* specific S1PR agonists/antagonists are available.

In conclusion, the present study demonstrated that FTY720 can prevent CCH-induced spatial memory impairment, oxidative stress, and neuroinflammation, possibly by activating SIRT3-independent mitochondrial pathway.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available. Requests to access the datasets should be directed to Miao Zhang, milkyzm@163.com.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Animal Experimentation of Wuhan University.

AUTHOR CONTRIBUTIONS

MZ and YH conceived the study and performed the experiments. JiZ analyzed the data. JuZ drafted the manuscript. All authors edited and approved the manuscript.

FUNDING

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Long-Term Prognosis of Cognitive Function in Patients With Idiopathic Normal Pressure Hydrocephalus After Shunt Surgery

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The long-term prognosis of cognitive function in patients with idiopathic normal pressure hydrocephalus (iNPH) remains unclear. This study aimed to determine the long-term prognosis of cognitive function in patients with iNPH, as well as the factors related to it. It included 48 patients with iNPH who were treated with cerebrospinal fluid shunting between January 2015 and December 2017 at Osaka Medical College Hospital, with follow-up evaluation of their cognitive function for >2 years. Cognitive function was measured using the Mini-Mental State Examination (MMSE) preoperatively and at 3 months, 1 and 2 years post-operatively. The mean MMSE score (22.4 ± 5.4 preoperatively) improved at 3 months [23.8 ± 5.0 ($p = 0.0002$)] and 1 year [23.7 ± 4.8 ($p = 0.004$)] post-operatively. At 2 years post-operatively, they were able to maintain their preoperative level (22.6 ± 5.3). The patients were classified in to the cognitive decline group [11 (23%) patients; a decrease in the MMSE score by ≥ 2 points 2 years after surgery] and the maintenance/improvement group [37 (77%) patients]. Univariate and receiver operating characteristic analyses were performed for the two groups to identify factors associated with cognitive prognosis. In both groups, the patients who were younger ($p = 0.009$) or had milder symptoms ($p = 0.035$) had a better long-term prognosis of cognitive function. The cutoffs for age and disease severity (idiopathic normal-pressure hydrocephalus grading scale; INPHGS) were 78 years (area under the curve = 0.77) and 5 points (area under the curve = 0.71), respectively. In conclusion, most patients (77%) were able to improve and maintain cognitive function for at least 2 years after surgery. The fact that disease severity and age are associated with cognitive prognosis suggests that early iNPH intervention is desirable to improve cognitive prognosis.

Keywords: hydrocephalus, cognition, dementia, shunting, long term outcome

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INTRODUCTION

Idiopathic normal pressure hydrocephalus (iNPH) is clinically characterized by gait disturbance, dementia, and urinary incontinence (Vanneste, 2000; Relkin et al., 2005). Cerebrospinal fluid (CSF) shunting can be used to treat it (Kuriyama et al., 2017). Currently, iNPH is considered a rare disease; however, several recent population-based epidemiological studies have shown that iNPH is common and affects 2–3% of older adults (Hiraoka et al., 2008; Tanaka et al., 2009; Andersson et al., 2019). Given that the progression of gait and cognitive impairment leads to the requirement of care at home and in the community, iNPH is becoming increasingly important with respect to public health and social security.

To reduce the caregiving burden on families and society, iNPH treatment for short- and long-term improvement and maintenance of gait and cognitive function is required. There have been numerous reports regarding the long-term prognosis of gait function after CSF shunt surgery in patients with iNPH. Gait function was found to be improved in ∼80% of cases at 3 years post-operatively; moreover, the long-term prognosis of gait function was good (McGirt et al., 2008; Pujari et al., 2008).

However, few studies have reported the long-term prognosis of cognitive function in patients with iNPH, with most studies having a follow-up period of about 1 year (Yamada et al., 2017). Even in the reports of long-term prognosis, there are no studies reporting the post-operative progression as measured using cognitive function tests, and the prognostic factors have not been clearly reported (Koivisto et al., 2016; Grasso et al., 2019). Consequently, the long-term cognitive prognosis, course of iNPH after 2 post-operative years, and their related factors remain unclear. This retrospective study aimed to determine the long-term cognitive trends and factors that affect long-term cognitive function in patients with iNPH after CSF shunt surgery.

MATERIALS AND METHODS

Eligible Patients

Out of the 72 patients with iNPH who were treated with CSF shunting from January 2015 to December 2017 at Osaka Medical College Hospital, we included 48 patients whose cognitive function could be evaluated with follow-up for >2 years.

The excluded cases included 10 deaths (6, 1, 1, 1, and 1 cases of pneumonia, bladder cancer, head injury, subarachnoid hemorrhage, and sudden death of unknown cause, respectively), 4 transfers, 10 cases of distance-related difficulties in accessing the hospital, and 1 case of difficulty in completing the Mini-Mental State Examination (MMSE).

The indications for surgery were according to the Japanese Idiopathic Normal Pressure Hydrocephalus Treatment Guidelines, 3rd edition. All patients with ≥1 gait, cognitive, or urinary deficit who presented with enlarged ventricles (Evans index >0.3) and lacked other neurological or non-neurological diseases that explained the aforementioned clinical symptoms or a previous history of any disease that could cause enlarged ventricles were enrolled (Relkin et al., 2005; Jaraj et al., 2017). CSF shunting was performed in patients with a positive CSF tap test and in those with a negative tap test but with disproportionately enlarged subarachnoid space hydrocephalus on magnetic resonance imaging (Mori et al., 2012).

Measurement Parameters

We retrospectively obtained the following measurement parameters: age, sex, idiopathic normal pressure hydrocephalus grading scale (INPHGS) (Mori, 2001), comorbidities, time to surgery, and surgical technique. A speech therapist administered the MMSE (Folstein et al., 1975) to the patients before the spinal tap test and at 3 months, 1 and 2 years after the CSF shunt surgery. The MMSE is useful as a screening tool for dementia and gives information about the severity of dementia.

The INPHGS consists of sections on gait disturbance, dementia, and urinary incontinence. Gait disturbance is defined as 0, normal; 1, unstable but independent gait; 2, walking with one cane; 3, walking with two canes or a walker frame; and 4, walking not possible. Dementia is defined as 0, within the normal range; 1, no apparent dementia but apathetic; 2, socially dependent but independent at home; 3, partially dependent at home; 4, totally dependent. Urinary incontinence is defined as 0, absent; 1, absent but with pollakiuria or urinary urgency; 2, present sometimes only at night; 3, present sometimes even during the day; 4, frequent. The grades of gait disturbance, dementia, and urinary incontinence were summated to obtain the total grade, which ranges from 0 to 12.

To investigate the factors associated with poor cognitive prognosis, the patients were divided into two groups: the maintenance/improvement group ($n = 37$) and the decline group ($n = 11$). A previous study has reported that a mean difference of 1.1 points was observed when the MMSE was administered twice, 24 h apart, by the same tester (Mori et al., 2012). Therefore, a one-point reduction was considered to be within the error range and the group that dropped more than 2 points was considered to be the decline group.

Statistical Analysis

Changes in the MMSE score were assessed using Wilcoxon's signed-rank test. Between-group differences in age, sex, comorbidities, time to surgery, operative technique, and disease severity were assessed using Pearson's chi-square test and Student's *t*-test. Parameters significantly associated with long-term cognitive function were entered into a logistic regression model to assess the cutoff values and area under the curve. Receiver operating characteristic curves were drawn to visualize the effect of these parameters on cognitive prognosis.

Moreover, factors associated with the prognosis of cognitive function were extracted using a decision tree analysis.

Data analyses were performed using JMP Pro 15.1. This software was developed by SAS in North Carolina, USA, in 1989 to help researchers and technicians visually analyze data.

RESULTS

Demographic and Clinical Data

Table 1 lists the patients' baseline characteristics. Their mean age was 76.9 (±5.8) years; there were 29 (60.4%) men

TABLE 1 | Demographic and clinical characteristics of the patients ($n = 48$).

Clinical characteristic ($n = 48$)	
Age, mean (SD)	76.9 (±5.8)
Sex, % (n)	
Men	60.4% (29)
Women	39.6% (19)
INPHGS, mean (SD)	5.3 (±2.3)
MMSE score, mean (SD)	22.4 (±5.4)
Days until surgery, mean (SD)	62.9 (±30.)
Type of surgery, % (n)	
LP shunt	81.2% (39)
VP shunt	18.8% (9)
INPHGS, % (n)	
Responder	89.6% (43)
Non-responder	10.4% (5)

The total scores of gait disturbance, dementia, and urinary incontinence were evaluated on a 12-point scale. Improvement (responder) was considered as a decrease in the total INPHGS score by least one point at 1 year after surgery, when the average MMSE score peaked, compared to the preoperative levels.
INPHGS, Idiopathic Normal Pressure Hydrocephalus Grading Scale; MMSE, Mini-Mental State Examination; LP, Lumboperitoneal; VP, Ventriculoperitoneal; SD, Standard deviation.

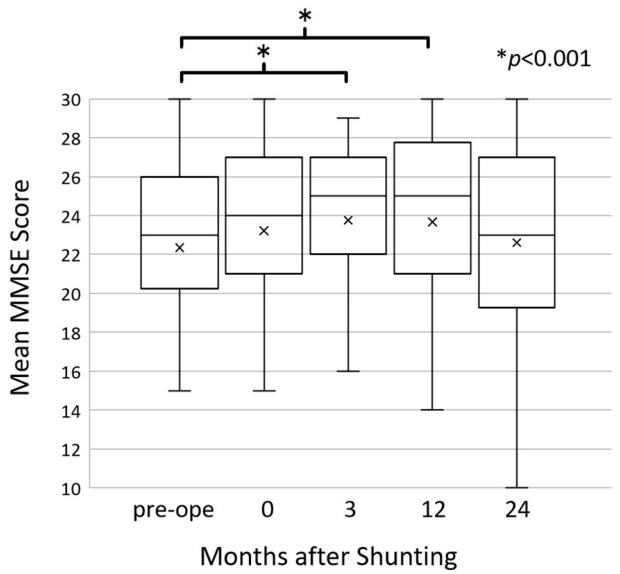

FIGURE 1 | The mean Mini-Mental State Examination (MMSE) scores before and after shunting. The mean MMSE scores improved at 3 ($p = 0.0002$) and 12 ($p = 0.004$) months post-operatively compared to the preoperative scores; however, they returned to the preoperative level at 24 months post-operatively. Changes in the MMSE score were assessed using Wilcoxon's signed-rank test.

and 19 (39.6%) women. The mean INPHGS score was 5.3 (±2.3) points. The average number of days from the tap test to surgery was 62.9 (±30) days. The surgical form was lumboperitoneal and ventriculoperitoneal shunting in 55.1 and 43.2% of patients, respectively. There were 43 (89.6%) patients who were administered the INPHGS; further, CSF shunting improved iNPH symptoms in most patients. In terms of major comorbidities, 6 patients had Alzheimer's disease (AD), 13 had hypertension, 12 had diabetes, 11 had hyperlipidemia, and 6 had cerebral infarction.

Changes in MMSE Over Time

There was a significant improvement in the MMSE score from 22.4 ± 5.5 preoperatively to 23.8 ± 5.0 ($p = 0.0002$) and 23.7 ± 4.8 ($p = 0.004$) at 3 months and 1 year post-operatively, respectively. At 2 years post-operatively, the MMSE score was maintained at the preoperative level (MMSE = 22.6 ± 5.3) (**Figure 1**).

At 2 years post-operatively, the MMSE scores increased by ≥2 points in 12 patients, decreased by ≥2 points in 11 patients, and showed almost no change in 25 patients. The maintenance/improvement group's MMSE score improved from 22.5 ± 5.7 to 24.2 ± 4.7 ($p < 0.0001$) (**Figure 2**). Conversely, the decline group's MMSE scores decreased from 21.9 ± 4.6 to 17.3 ± 4.0 ($p = 0.0002$).

Cognitive Prognostic Factors

There were 37 (77%) and 11 (23%) patients in the maintenance/improvement and decline groups, respectively. Student's *t*-test revealed significant between-group differences in age ($p = 0.009$) and INPHGS ($p = 0.035$) (**Table 2**). However, there were no significant between-group differences in time to surgery ($p = 0.863$), sex ($p = 0.804$), and surgical technique ($p = 0.956$). Logistic regression analysis revealed that the cutoffs for age and INPHGS score were 78 years (area under the curve = 0.77) and 5 points (area under the curve = 0.71), respectively (**Figure 3**). Receiver operating characteristic curve analysis revealed that poor prognosis was associated with old age and preoperative symptom severity.

In a decision tree analysis for increasing the predictability of maintenance/improvement, age and INPHGS score were selected as the first and second nodes, respectively (**Figure 4**). Here, a preoperative age of <79 years and INPHGS score of <4 points had the best outcome (100% probability of maintenance/improvement) while a preoperative age of ≥79 years and INPHGS score of ≥7 had the worst outcome (29% probability of maintenance/improvement).

DISCUSSION

In this study, 77% of the patients with iNPH showed improved and maintained cognitive function after CSF shunting for at least 2 years post-operatively. This is the first study to perform a systematic evaluation of cognitive function for >2 years. Previous studies have reported that the MMSE scores of patients with iNPH improved by about 1 point within 1 year; however, it remained unclear whether this recovery was subsequently maintained (Solana et al., 2012; Shaw et al., 2016). Despite examining a small number of patients, some studies have reported no long-term improvement in cognitive function after shunting (Mori, 2001; Savolainen et al., 2002; Kahlon et al., 2007; McGirt et al., 2008). We found that the MMSE score improved

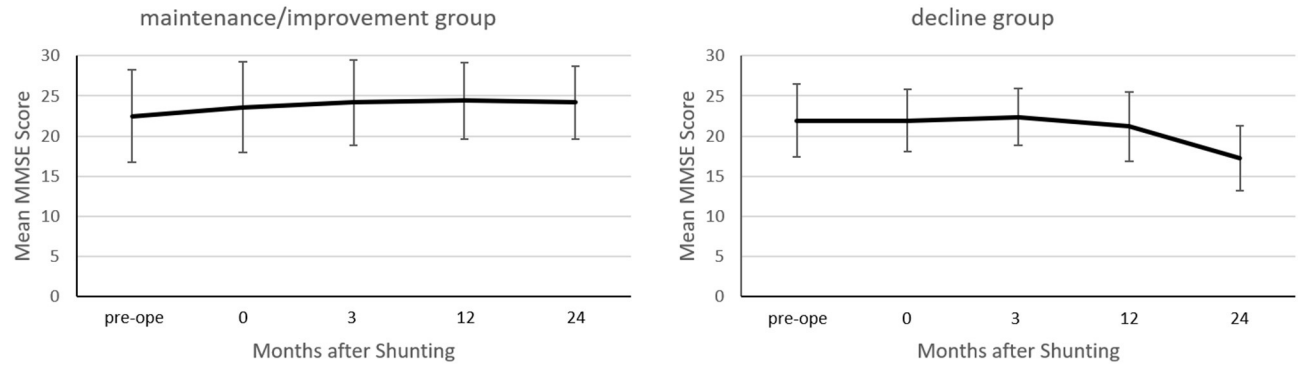

FIGURE 2 | Between-group comparison of the mean Mini-Mental State Examination (MMSE) score before and after shunting. In the maintenance/improvement group, the mean MMSE score improved at 24 months post-operatively compared to preoperatively ($p < 0.0001$). Conversely, in the decline group, the mean MMSE score declined at 24 months post-/operatively compared to preoperatively ($p = 0.0002$). Changes in the MMSE score were assessed using Wilcoxon's signed-rank test.

TABLE 2 | Between-group comparisons of the clinical data.

	Maintenance/ improvement group ($n = 37$)	Decline group ($n = 11$)	*p*-value
Age, mean (SD)	75.7 (±5.8)	80.8 (±3.7)	0.009*
Sex, % *(n)*			
Men	59.5% (22)	63.6% (7)	0.804
Women	40.5% (15)	36.3% (4)	
INPHGS (preoperatively), mean (SD)	4.9 (±2.2)	6.5 (±2.1)	0.035*
Days until surgery, mean (SD)	63.3 (±33.0)	61.6 (±17.1)	0.863
Type of surgery, % *(n)*			
LP shunt	81.1% (30)	81.8% (9)	0.956
VP shunt	18.9% (7)	18.2% (2)	

*INPHGS, Idiopathic Normal Pressure Hydrocephalus Grading Scale; LP, Lumboperitoneal; VP, Ventriculoperitoneal; SD, Standard deviation. *p-value was calculated using Pearson's χ^2 test for categorical variables and Student's t-test for continuous variables with normal distribution.*

by an average of 1.2 points at 1 year post-operatively and nearly returned to preoperative levels at 2 years post-operatively. Specifically, this indicates that cognitive function is maintained for 2 years.

Generally, iNPH is a progressive disease. Patients with untreated iNPH showed a decrease in the MMSE score by 3 points at 13 months after the diagnosis of iNPH (Andrén et al., 2014). The maintenance of cognitive function for >2 years indicates that CSF shunting has a significant therapeutic effect on cognitive function in patients with iNPH.

Although long-term cognitive prognosis varies widely across individuals, the predictive factors include age ($p = 0.009$) and INPHGS ($p = 0.035$). Future studies should determine the prediction accuracy.

This study included a relatively large number of older patients, with a mean age of 75.7 ± 5.8 and 80.8 ± 3.7 years in the maintenance/improvement and decline groups, respectively. Although the etiology of iNPH remains unclear, aging is considered as its greatest risk factor. More than 50% of patients with iNPH develop other neurodegenerative diseases. Therefore, age could be associated with long-term poor prognosis of cognitive function, since older age increases the probability of comorbidities (Golomb et al., 2000; Leal et al., 2018; Libard et al., 2018). In the short term, comorbid AD does not affect the post-operative improvement of iNPH symptoms; however, there have been no long-term follow-up studies (Bech-Azeddine et al., 2007).

In this study, six patients had a history of AD, and one patient developed AD during the follow-up period. Of them, one patient showed an improved MMSE score after the shunting; however, there was a gradual cognitive decline after 1 year. Further, the patient was diagnosed with post-operative AD based on symptoms and a single-photon emission computed tomography scan showing hypovolemia in the bilateral posterior cingulate gyri. After 2 years, the patients diagnosed with AD had a greater decrease in the MMSE score (by 11 and 5 points, respectively) than those without AD. In addition to AD, cerebral vascular disease has been reported to be highly comorbid with iNPH (Bech-Azeddine et al., 2007). None of the participants experienced cerebral infarction within 2 years of the surgery. Two of the six patients with a history of cerebral infarction had a decrease MMSE score (by 11 and 3 points, respectively). However, all patients did not undergo close examination for dementia complications, including AD, during the follow-up period. Therefore, there is a need to detect the involvement of other diseases for appropriate iNPH treatment.

Regarding the comorbidities of iNPH, CSF circulation in the glymphatic pathway has recently been shown to be associated with amyloid production in the brain (Mortensen et al., 2019). CSF amyloid and p-tau levels are reportedly increased post-operatively in patients with iNPH (Moriya et al., 2015).

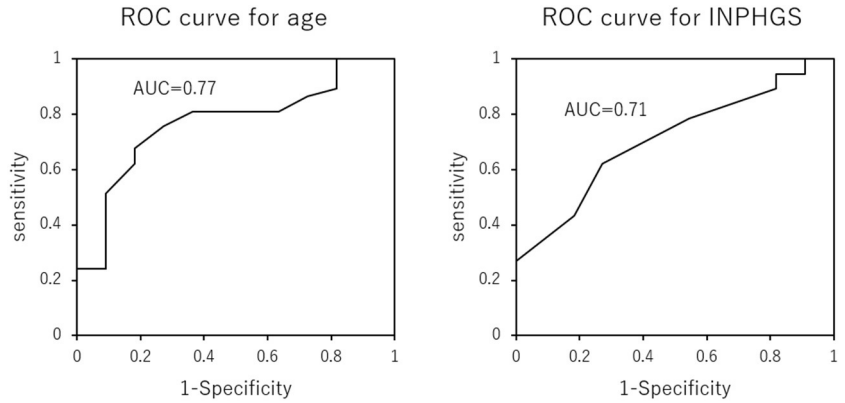

FIGURE 3 | Receiver operating characteristic curves for the effect of age and Idiopathic Normal Pressure Hydrocephalus Grading Scale score on long-term cognitive prognosis after shunting. Older age and more severe symptoms predicted a poor cognitive prognosis 2 years after surgery.

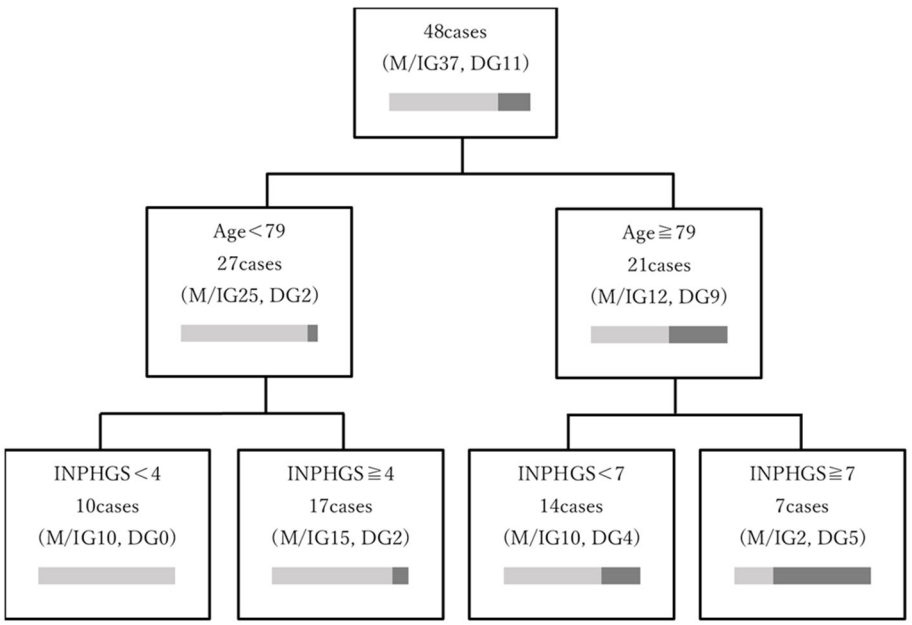

FIGURE 4 | Decision tree model depicting the prognostic factors associated with long-term cognitive function after surgery for idiopathic normal pressure hydrocephalus. The decision tree was based on age and Idiopathic Normal Pressure Hydrocephalus Grading Scale (INPHGS) score as variables related to long-term cognitive function and ordered by the relative importance of each feature included in the model. The characteristics associated with the most favorable outcome (100% probability of maintenance/improvement) were age <79 years and INPHGS score <4 points. Furthermore, the combination of preoperative age ≥79 years with INPHGS score ≥7 showed the worst outcomes (29% probability of maintenance/improvement).

Consequently, patients with iNPH may have progressive amyloid and p-tau accumulation resulting from impaired CSF circulation, which may increase the incidence of comorbidity, including AD (Reeves et al., 2020). Our finding that as many as 77% of patients showed improved and maintained cognitive function for 2 years after surgery could be indicative of inhibition of the pathophysiology of dementia itself.

This study shows that younger age and less severe iNPH contribute to a favorable long-term prognosis of cognitive function. This indicates the importance of early diagnosis and treatment for the long-term maintenance of cognitive function. Moreover, Andrén et al. (2014) reported that iNPH symptom progression is irreversible, and that delaying surgery could worsen symptoms. This indicates the need for early diagnosis and treatment to improve long-term cognitive prognosis in patients with iNPH.

This study has several limitations. First, the duration of this study was short, and future studies with longer follow-up periods are needed to validate our findings. We are currently designing a more than 3-year follow-up study that will include the participants of this study. Second, the MMSE, a screening test that can assess overall cognitive function, has limited utility in assessing cognitive function in iNPH, especially when used alone. In this study, the cognitive function of most patients is

at the level of mild cognitive impairment. A cognitive function test suitable for mild cognitive impairment, such as the Montreal Cognitive Assessment, is considered to have better accuracy (Nasreddine et al., 2005). We should use multiple tests to assess a wide range of cognitive functions. Finally, although we incorporated multiple predictors, not all potentially relevant predictors were incorporated. For example, future studies should consider including iNPH-specific neuroimaging parameters (e.g., Evans index) and factors associated with AD and atherosclerosis. However, for comorbidities, the diagnosis of AD and other comorbidities may require pathological examination, which was ethically difficult to clarify in all cases because of the need for invasive procedures. In conclusion, patients with iNPH showed significantly improved cognitive function for 3 months to 1 year after CSF shunt surgery, which was maintained until at least 2 years. Cognitive function improvement and maintenance were more pronounced in younger patients with milder disease. Therefore, early diagnosis and prompt treatment are important for long-term improvement and maintenance of cognitive function in patients with iNPH.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Osaka Medical College. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AK, YK, and MW made substantial contributions to the conception and design of the study. AK, YK, KK, ST, and RS collected data regarding the participants and task performance. AK, YK, RY, NI, MF, NN, SK, and TK analyzed the data. AK and YK wrote the manuscript. RS and MW supervised this project. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.617150/full#supplementary-material

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Rehabilitation and Disability Spectrum From Adverse Childhood Experience: The Impact of the Movement Cognition and Narration of Emotions Treatment (MCNT) Version 2.0

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Adverse Childhood Experiences (ACE) are associated with an increased risk of cerebral, behavioral, and cognitive outcomes, and vulnerability to develop a Borderline Intellectual Functioning (BIF). BIF is characterized by an intelligence quotient (IQ) in the range 70–85, poor executive functioning, difficulties in emotion processing, and motor competencies. All these difficulties can lead to mental and/or neurodevelopmental disorders that require long-term care. Accordingly, we developed an intensive and multidomain rehabilitation program for children with ACE and BIF, termed the Movement Cognition and Narration of emotions Treatment (MCNT1.0). The efficacy of MCNT1.0 on cognitive and social functioning was demonstrated with a previously reported randomized controlled trial (RCT). To extend the impact of the treatment also to the motor domain a new version, called MCNT2.0, was implemented. The present study aims to verify the feasibility of MCNT2.0 and its effects on the motor domain. A quasi-experimental approach was used in which a group of 18 children with ACE and BIF were consecutively recruited and participated in the MCNT 2.0 program. Participants were compared with the MCNT1.0 group as an active comparator, using the dataset of the RCT. The two groups received a full evaluation comprising: the Wechsler Intelligent Scale for Children-IV (WISC-IV), the Movement-ABC (M-ABC), the Test of Gross Motor Development (TGMD), the Social Skills from Vineland Adaptive Behavioral Scale-II (VABS-II) and the Child Behavior Check List 6–18 (CBCL). An ANCOVA was carried out on changes in the scale scores from baseline with age and baseline score as covariates. Results showed a mean adherence to treatment of 0.85 ($sd = 0.07$), with no differences between groups in IQ, and Social Skills changes, while greater improvements for motor abilities were shown in the MCNT 2.0 group: M-ABC ($p = 0.002$), and TGMD ($p = 0.002$). Finally, greater improvement in

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the CBCL scale was observed in the MCNT 1.0 group ($p = 0.002$). Results indicate that due to its positive effects on cognitive, social participation and motor domains, MCNT2.0 may represent a protective factor against maladaptive outcomes of children with ACE and BIF.

Keywords: adverse childhood experience, cognitive-behavioral and motor impairment, borderline intellectual functioning, multimodal rehabilitation, stressful environment, emotional deregulation

INTRODUCTION

Adverse environmental conditions are frequently associated with neuropsychiatric consequences during early age. Some studies suggested how the exposure to adverse childhood experience (ACE) contributes to altered structure and function in several neurobiological systems, mostly related to the limbic system, with a consequent "latent vulnerability" to multiple forms of youth and/or adult psychopathology (1–3). ACE can be defined as experiences requiring "significant adaptation by an average child" (4) and include "harms that affect children directly (e.g., abuse and neglect) and indirectly through their living environments (e.g., parental conflict, substance abuse, or mental illness)" (5). Borderline intellectual functioning (BIF) is an important and frequently unrecognized comorbid condition (6–9) with an increased risk of exposure to ACE compared to their peers (10) such as inadequate housing, low parental education, low social class, low income, absence of a parent, parental psychiatric morbidity (10). BIF is defined as a boundary condition between typical development and intellectual disability, characterized by an intelligence quotient (IQ) within the range 70–85, associated with difficulties in social participation and adaptability, and is recognized as a V code in the Diagnostic Statistical Manual-5 (11). Children with BIF exhibit difficulties in several developmental domains such as cognition, affectivity, sociality, and movement: learning disorders, impairment in executive functioning, receptive and expressive language, motor planning, emotional regulation, Theory Of Mind and behavioral difficulties are often detected (7, 12–18). Interestingly, children with BIF also exhibit a peculiar pattern of sleep organization characterized by an alteration of the cyclic alternating pattern, and a positive correlation between sleep duration and intellectual abilities (19, 20). In line with such evidence, several studies found a significant correlation between ACE and sleep disorders (21).

It has been shown that deprived environments negatively impact working memory, inhibitory control, cognitive flexibility (22–27), language (28, 29), and global intelligence (30). Accordingly, a recent study of a large cohort of 14,000 children showed that by the age of 2 years, children belonging to a low socio-economic environment had a 6 point lower IQ compared to their high socio-economic peers. This difference almost tripled when the same subjects were evaluated at the age of 16 (31). Finally, unpredictable and potentially threatening environments negatively impact the development of the emotional response and regulation systems with consequences on behavior and social relationships (4).

Children with ACE and BIF are thus a highly vulnerable population at risk of maladaptive outcomes, such as lifetime cognitive and mental disorders, anxiety, depression, substance abuse, externalizing/internalizing behavioral disorders, drop-out from schooling, and low income if left untreated.

To prevent the psychopathological drift following ACE and to respond to the several special needs of these children, we developed a rehabilitative intervention, termed the Movement Cognition and Narration of emotions Treatment [MCNT; (32)], which aimed at improving global intelligence, movement abilities and adaptive competences. MCNT lasts 9 months and consists in 3 h a day, 5 days a week of 3 laboratories: the Movement Lab, to improve fine and gross motor abilities; the Cognitive Lab, to improve reasoning, mental flexibility and wider executive functioning; and the Emotion Lab, to improve emotion recognition, comprehension and expression [for a detailed description of MCNT see the study protocol published (32)]. Indeed, this method targets the motor, cognitive and affective domains.

The efficacy of this intensive and multidomain experimental approach was investigated with a randomized controlled trial [RCT; (33)]. Results demonstrated that MCNT, which we shall rename here MCNT 1.0, was more effective than Standard Speech therapy (SST, usual care) in improving intellectual, adaptive and behavioral functioning in children with ACE and BIF, whereas no significant improvement was observed in motor abilities.

Moving from our previous results, we have focused on an *ad hoc* adjustment of the MCNT method, modifying the Movement Lab. We shall rename this new version as MCNT 2.0, in which we have abandoned the game therapy approach in the Movement Lab in lieu of a method focused on movement and body awareness, the Body Minding.

In the light of our previous results, which showed a poor effect of SST on cognitive and adaptive functioning, we preferred not to carry out a RCT with the SST as a control group but to directly compare the two versions of the MCNT intervention. Indeed, a treatment with poor efficacy might prevent the gain of competences relevant for children's development. Moreover, RCTs are expensive and not easy to implement in a routine care setting. For this reasons, we designed a quasi-experimental study (34) in which a group of 18 children with ACE and BIF were consecutively recruited to participate in the MCNT 2.0 program. This group was then compared with the children treated with MCNT 1.0 from the original RCT [(32, 33)].

The aims of the current study were to evaluate the feasibility of MCNT 2.0 intervention and its effect on motor skills in a sample of children with ACE and BIF. Based on our previous

experience, we expected MCNT 2.0 to be feasible, and that compared to MCNT 1.0, the newer implementation would reveal a significant positive effect on motor abilities, without any differences between the two interventions on cognitive, social, and behavioral competencies.

MATERIALS AND METHODS

Study Design and Participants

The Study was approved by the Ethics Committee of the Don Gnocchi Foundation (DGF) and of the ASST S. Paolo and S. Carlo Hospital. All parents signed a written informed consent at the first meeting.

This was a quasi-experimental study in which a group of children with BIF and exposed to ACE were consecutively recruited and treated with MCNT 2.0, with all the children belonging to the group treated with MCNT 1.0 as an active comparator, consisting of 18 subjects that underwent the rehabilitation intervention in the Years 2016–2017. The MCNT 1.0 group belongs to the dataset of our previous RCT (33).

Participant assignment in the MCNT 2.0 group was not randomized. However, to support internal validity and to reduce sample differences, participants were selected from the same catchment area of the city of Milan and with the same inclusion/exclusion criteria of our previous RCT.

Inclusion criteria were: age range between 6–11 years old and attending primary mainstream school; a Full Scale Intelligence Quotient (FSIQ) score ranging from 70 to 85; presence of an impact on social functioning (school and/or family context) as derived from the clinical history. Since all children participating in the MCNT 1.0 program belonged to a middle, middle-low or low socio-economic status [SES <39, (35)], we wanted the two groups to be matched for this parameter to control for confounding variables that may threaten the internal validity of the study. For this reason, we verified that none of the children of the MCNT 2.0 were of a high SES background.

Exclusion criteria were: presence of major neuropsychiatric disorders (such as ADHD and autism spectrum disorder); presence of neurological conditions such as epilepsy, traumatic brain injury, brain malformation and infectious disease involving the central nervous system. Other exclusion criteria considered were: the presence of systemic diseases such as diabetes or dysimmune disorders, genetic syndromes such as Down syndrome or Fragile X syndrome. Furthermore, a positive history for psychoactive drugs, particularly referring to current or past use of psychostimulants, neuroleptics, antidepressants, benzodiazepines, and antiepileptic drugs were also considered exclusion criteria.

The MCNT 2.0 group consisted of 18 children with ACE associated with BIF (age: mean = 7.68; $sd = 1.25$) and attending mainstream primary school in Italy treated with MCNT 2.0 (see methods section) in the years 2018/2019. Initially, 19 participants were enrolled but one child abandoned the study and was excluded from analyses. The MCNT 1.0 group consisted of 18 children with ACE and BIF (mean age = 7.78; $sd = 1.31$).

Characteristics of two groups at baseline are presented in **Table 1**.

TABLE 1 | Demographic and baseline data.

Variable	MCNT$^{1.0}$	MCNT$^{2.0}$	Group comparison (*p*-value)
Subjects (Number)	18	18	
Sex (M:F)	8:10	7:11	1.0
Age (Years, Mean ± *SD*)	7.78 ± 1.31	7.67±1.28	0.83
SES* (Mean ± *SD*)	24.03 ± 11.64	21.69 ± 9.95	0.34
ESCL* (Mean ± *SD*)	3.67 ± 2.85	4.22 ± 3.14	0.55
WISC-FSIQ (Mean ± *SD*)	75.11 ± 8.52	77.22 ± 5.33	0.84
Adherence to treatment	0.88 ± 0.07	0.84 ± 0.07	0.09

*MCNT, Movement Cognition and Narration of the emotions Treatment 1.0 and 2.0 version; SES, Socio-Economic Status; ESCL, Environmental Stress Checklist; WISC, Wechsler Intelligent Scale for Children; FSIQ, Full Scale IQ; *, the variable was not normally distributed and thus the Mann-Whitney test was used.*

Clinical Assessment on ACE, Motor, Cognitive, and Behavioral Domains

To detect the presence of ACE we used the Environmental Stress Check-List [ESCL; (2)]. The ESCL consists in a listing of the V-codes from DSM-5, and Z-codes from ICD-10, that explore problems related to relational, neglect, physical, sexual and/or psychological abuse, educational and occupational, housing and economic, social exclusion or rejection, plus the presence of social services intervention, of major psychiatric diagnosis, and of substance abuse within the family members. A 0 (absence) to 1 (presence) score was attributed to each item after careful consideration of its relevance for the clinical manifestations. The ESCL total score ranges from 0 to 24 with higher values indicating a greater number of environmental stressful conditions.

The clinical assessment on motor, cognitive and behavioral domains was carried out at two time points (T0, within 2 months prior to the beginning of the treatment and after 9 months at T1 within 2 months after the end of the treatment).

Motor domain was assessed by a Neuro-Psychomotor in Developmental Age Therapist using:

1. The Movement-ABC [M-ABC; (36)], for the assessment of the motor skills, included manual dexterity, ball skills and static/dynamic balance; the total score was expressed in percentiles and fall into clinical range for scores below 6th percentile, into borderline range from 6th to 15th percentile and into normal range above 15th percentile;
2. The Test of Gross Motor Development [TGMD; (37)] through the assessment of both Locomotor and Object Control abilities give a measure of gross motor skill development, the Gross Motor Quotient (GMQ);

Cognitive domain was assessed by a neuropsychologist using:

1. The Wechsler Intelligence Scale for Children-IV [WISC-IV; (38)] to measure global intellectual functioning as full scale IQ (FSIQ) and the following indices: verbal comprehension index (VCI); perceptual reasoning index (PRI); working memory index (WMI); processing speed index (PSI). Children belonging to the MCNT 1.0 group were evaluated with the

previous version of the scale, the WISC-III (39). Despite substantial differences between the two versions in the structure (the later revision of the Scale removed three and introduced five new subtests modifying its structure) and the construct of the indices and of the FSIQ, there is a very high correlation (0.89) in the FSIQ between the two versions, as previously reported (40). Indeed, we decided to use the FSIQ for the between group comparison because it is more stable and reliable when compared to the indices scores across the two versions of the WISC scale. Moreover, to avoid direct comparison of the scores of the two versions, we used delta values (post-pre-treatment scores), a longitudinal single subject change over time approach, to compare the two groups of children.

Behavioral and social skills were assessed by a psychologist using:

1. The Vineland Adaptive Behavioral Scale II [VABS-II; (41)], to assess social functioning, communication abilities, daily living skills, and a full scale quotient (FSQ) of adaptive functioning through a single interview with parents.
2. The Child Behavior Checklist 6–18 [CBCL 6–18; (42, 43)], in the Parent's Report Form, to evaluate emotional and behavioral problems in children and adolescents; data were expressed in Tscore and higher scores indicate greater problems; the total score can be interpreted as falling in the normal (<60 Tscore), borderline (60–63 Tscore), or clinical range (>63 Tscore).

Intervention: the MCNT 2.0

The MCNT2.0 is an adapted version of MCNT1.0, an intensive and multimodal rehabilitation program, whose effectiveness has been demonstrated in our previous study (33). A detailed description of this method is present in the study protocol (32). MCNT consists of three laboratories (Lab), the Movement Lab, the Cognitive Lab and the Emotion Lab, in which children work in small group of seven to eight. Due to the lack of significant results, the Movement Lab was the only one that underwent substantial adjustment from MCNT 1.0–2.0.

Briefly, the first laboratory, the Cognitive Lab, aims at cognitive empowerment working on executive skills, such as fluid reasoning, problem solving, attention, inhibitory control, monitoring, switching, and academic competencies (reading, writing, and calculating) including listening comprehension with the use of the multimedia interactive whiteboard (MIW). The second lab, the Emotion Lab, adopts a relational dynamic approach to improve emotion expression, recognition, comprehension, and autoregulation. Purpose of the Emotion Lab is the "alphabetization" of the emotions (44). Spontaneous play, drawing, stories (invented and/or dramatized) and talking are the preferred tools used by the psychotherapist to achieve these goals. Finally, the third laboratory, the Movement Lab, fostered the improvement of global motor functioning with an game therapy approach using commercial gaming consoles. In the MCNT 2.0 the game therapy was substituted with a method focused on movement and body awareness. The focus was the body as a means through which we move in space and experience feelings. In more details, the Movement Lab 2.0 included two types of activities: (1) bodily movements to work on the "acting self" (45, 46) that is the sense of agency of own body during an action and include feelings and movement controls. This activity included sequences of coordination movements, from the simplest to the most complex, that involved bimanual and interlimbic coordination patterns but also eyes, ears, tongue, breath, and other cross movement designed for stimulating hemispheric brain interconnection and improving balance, stability, coordination and planning, speed, and accuracy in the movements. During the performance, children's attention was usually driven on "feeling the body." Common games such as jumping rope, hopscotch, target shooting, balance play were performed to increase coordination, speed and accuracy; and (2) bodily awareness/perception to work on the "sensorial self" that is the sense of ownership of body and perceptual experience of all body parts in the external space (45–47). The child was guided toward the representation of his/her body through relaxation techniques guided by imaginative processes.

The MCNT 2.0 treatment was carried out for 9 months in a hospital setting. Children were accompanied from school to our Center with a shuttle service provided by our Institution. Children attended all the three Labs every day for 5 days/week, 3 h/day in the afternoon, working in small groups. For each group two specialized operators were assigned. Moreover, weekly meetings among professionals were carried out to monitor treatment, and to discuss emerging difficulties. Finally, at least two meeting with teachers, and at least 5 meetings with children's parents were provided. The number of the meetings was based on the specificity of each child situation. These meetings had the objective to create a support network and discuss the methodology of the intervention, evaluate the specific needs of each child, and find solutions to problems as they arose.

Adherence to treatment for each participant was calculated as the number of attended sessions divided by the number of total sessions and used as a measure of feasibility.

Statistical Analysis and Sample Size Calculation

Since no preliminary data relative to motor abilities from our lab were available, the sample size was calculated according to data from the literature (48). The *a priori* sample size calculation was performed with G*Power software 3.1, considering a medium effect size (Cohen's f) = 0.25 (49), with an expected power of at least 0.80 and an alpha value 0.05. According to this procedure the estimated sample size *a priori* was 34.

Statistical analysis was conducted using SPSS software (version 24). Before proceeding to hypothesis testing, we checked the normal distribution for all measures using both the Kolmogorov-Smirnov and Shapiro-Wilk tests. A parametric (one-way ANOVA) or non-parametric (Mann-Whitney) comparison was performed as appropriate to compare baseline demographic and clinical characteristics of the two groups of children, MCNT 1.0 and MCNT 2.0. A chi-squared was used to test differences between groups for sex.

The measures considered in this study were changes in the scale scores from baseline (delta scores). For variables not normally distributed, a Bloom's transformation was applied to normalize scores. An analysis of covariance (ANCOVA), with age and pre intervention evaluation score as covariates, was carried out. Score differences were described using estimated mean, mean difference, and R^2 model fitting. An α value of 0.05 was considered statistically significant, and all comparisons were 2-tailed. The false discovery rate (FDR) correction was used to adjust for multiple comparisons between the different measures (50).

The magnitude of effects was calculated and reported with effects size η^2 interpreted as follows: 0.01 as a small effect; 0.06 as an intermediate effect; 0.140 and higher as a strong effect (51).

Except for M-ABC and CBCL all data were expressed in standard score.

Data relating to the Communication scale, Daily Living Skills and FSQ of VABS-II and the four indices of WISC-IV were investigated with a paired *t*-test in the MCNT 2.0 group only.

RESULTS

Demographics and Feasibility

Table 1 shows baseline comparison between MCNT 1.0 and 2.0 groups. The two groups were matched for sample size, age, sex, SES, ESCL, and FSIQ.

Adherence for MCNT 1.0 was 0.88 ($sd = 0.07$), for MCNT 2.0 was 0.84 ($sd = 0.07$) with no statistical difference (**Table 1**). Both groups showed a very small number of drop-outs: 2 children in the MCNT 1.0 (one child moved to another country before post treatment evaluation, while another child had difficulties in working in a group setting) and 1 child in the MCNT 2.0 group (home to hospital distance was too great for the family to manage).

A detailed listing of the prevalence of each environmental stressor for each group was illustrated in **Table 2**.

Between Group Comparison

To determine the effects of MCNT 2.0 on motor, cognitive and behavioral/social competencies, an ANCOVA analysis was performed. Delta values (post-pre-treatment) of each scale were compared between groups with age and baseline evaluation as covariates.

Results are reported in **Table 3**. To summarize, the MCNT 2.0 group showed greater motor ability improvements compared to MCNT 1.0, as detected by both scales: M-ABC ($p = 0.002$, before Bloom's transformation delta values for MCNT 1.0 mean $= 7.85$, $sd = 15.19$; MCNT 2.0 group mean $= 40.95$, $sd = 21.89$), and TGMD ($p = 0.002$). For cognitive abilities detected with the FSIQ, and for Social Skills as detected by the VABS II scale, no significant differences were detected between the two groups. For behavioral competencies, detected with the CBCL scale, a greater improvement was observed in the MCNT 1.0 group ($p = 0.002$).

Within MCNT 2.0 Group Comparison

To better detail the changes in the intellectual and adaptive functioning of the MNCT 2.0 group only, a paired *t*-test was performed on pre vs. post treatment scores.

Results are reported in **Table 4**. Data showed significant difference between pre and post treatment scoring for Perceptual Reasoning Index (PRI, $p = 0.04$) within the WISC IV evaluation, and for Communication ($p = 0.04$), Daily Living Skills ($p = 0.04$), and the Full-Scale Quotient ($p = 0.04$) within the VABS II.

DISCUSSION

In this work we report data showing the feasibility in term of treatment adherence of the MCNT 2.0 and its greater effects on motor abilities compared to the previous version of the treatment, the MNCT 1.0.

The previous version of the MCNT treatment, MCNT 1.0, was investigated with an RCT study (33) whose results showed a positive effect of the treatment on intellectual, social and behavioral competences in comparison to standard care (i.e., individual speech therapy). Despite MCNT 1.0 having implemented interventions targeting motor competences, significant improvement was not detected in this regard. For this reason, an adjusted version of MCNT was created, in which the movement training component was modified with the introduction of the Body-Minding, a body awareness and interlimbic coordination program; this version was re-named MCNT 2.0. Accordingly, first aim of this study was to evaluate the feasibility and the effects of this modification on the motor competencies. To this purpose, we conducted a quasi-experimental study in which data relative to a non-randomized group of children was compared to a secondary dataset from the previously mentioned RCT (34, 52).

The first result is the feasibility of the MCNT 2.0. Despite the intensity and the long duration of the treatment, we observed a very high adherence coupled with a very low number of drop-outs, demonstrating the feasibility of the MCNT 2.0 program with no differences with the original treatment. In both versions, a shuttle service accompanied children from school to our Institution. Moreover, both versions of the MCNT provided support for the participant's teachers and their families. Having facilitated the access to our Institution and having provided support to the schools enabled the creation of a network supporting children and their families that we believe explains such a high adherence to the rehabilitation treatment.

Another important result of the present study is the confirmation that the MCNT method is effective in the improvement of the intellectual abilities of children with BIF. This is in line with the results of the previous RCT study (33). Specifically, no difference between the two groups was observed in the changes observed after treatment. The two groups showed similar improvement in the full-scale IQ. Moreover, data from the MCNT 2.0 group, showed that this datum was likely due to the increment in the Perceptual Reasoning, as shown by the increment in the PRI. These data are in agreement with the results of our previous study

TABLE 2 | Environmental Stress Check List scoring.

	MCNT 1.0 *N* of subjects	MCNT 2.0 *N*. of subjects
V60.1 (Z59.1)—Inadequate Housing	3	2
V60.2 (Z59.6)—Low income	10	5
V61.03 (Z63.5)—Disruption of family by separation or divorce	2	5
V61.20 (Z62.820)–Parent-child relational problem	3	7
V61.21 (Z69.010)—Encounter for mental health services for victim of parental child physical abuse	2	3
V61.21 (Z69.020)—Encounter for mental health services for victim of non-parental child physical abuse	0	0
V61.21 (Z69.010)—Encounter for mental health services for victim of parental child sexual abuse	0	0
V61.21 (Z69.020)—Encounter for mental health services for victim of non-parental child sexual abuse	2	0
V61.21 (Z69.010)—Encounter for mental health services for victim of parental child neglect	4	0
V61.21 (Z69.020)—Encounter for mental health services for victim of non-parental child neglect	0	0
V61.21 (Z69.010)—Encounter for mental health services for victim of parental child psychological abuse	0	3
V61.21 (Z69.020)—Encounter for mental health services for victim of non-parental child psychological abuse	0	2
V61.29 (Z62.898)—Child affected by parental relationship distress	2	2
1.V61.8 (Z62.891)—Sibling Relational problem	0	1
V61.8 (Z62.29)—Upbringing away from parents	0	2
V61.8 (Z63.8)—High expressed emotion level within family	6	2
V62.3 (Z55.9)—Academic or educational problem (Underachievement in school)	17	18
V62.3 (Z55.9)—Academic or educational problem (School-Family conflicts)	1	5
V62.4 (Z60.3)—Acculturation difficulty	4	5
V62.4 (Z60.4)—Social exclusion or rejection	2	1
(Z63.2)—Inadequate family support	3	5
(Z63.3) Absence of family member	0	4
Social Services Intervention	4	2
Major Psychiatric Diagnosis within the family	1	0
Substance abuse within the family	0	2

Prevalence of Environmental Stress factors in the group MCNT 1.0 and MCNT 2.0.

TABLE 3 | ANCOVA analysis of changes from the baseline, with age and baseline score as covariates.

Scale	MCNT 1.0 Estimated Mean (St. Err.)	MCNT 2.0 Estimated Mean (St. Err.)	MCNT 1.0 vs. MCNT 2.0 Mean Difference (St. Err.)	*F*	*P*-value (FDR)	η^2	ω	Adj. R^2
FSIQ	10.21 (2.56)	7.57 (2.56)	2.65 (3.64)	0.53	0.473	0.016	0.109	0.037
M-ABC*	−0.74 (0.23)	0.54 (0.19)	−1.27 (0.33)	15.28	**0.002**	0.361	0.965	0.381
TGMD	2.99 (2.55)	21.17 (2.39)	−18.18 (3.72)	23.95	**0.002**	0.444	0.997	0.422
CBCL	−10.32 (1.86)	2.19 (1.86)	−12.51 (2.66)	22.17	**0.002**	0.460	0.995	0.600
Social-VABS II	9.53 (2.44)	5.47 (2.22)	4.06 (3.50)	1.35	0.325	0.063	0.197	0.395

*MCNT, Movement Cognition and Narration of the emotions Treatment 1.0 and 2.0 version; St. Err, Standard Error; WISC III/IV, Wechsler Intelligent Scale for Children; FSIQ, Full Scale IQ; M-ABC, Movement Assessment Battery for Children (data expressed in percentiles); *, due to non-normal distribution of this variable a Bloom's was transformation applied; TGMD, Test of Gross Motor Development; TGMD, Test of Gross Motor Development; VABS-II, Vineland Adaptive Behavior Scale II; FDR, False Discovery Rate correction; ω, power; η^2, Partial Effect Size; Significant results are reported in bold font.*

(33) in which an increment in the Performance Quotient was observed in the MCNT 1.0 group. Both indices are measures of Fluid Reasoning (Gf), Comprehension-Knowledge (Gc), and Visual Processing (Gv). The metacognitive strategies used into the Cognitive Lab, which remained unmodified compared to the previous version, facilitated the empowerment of creative thinking through the exploration of new solutions, different perspectives, brainstorming techniques, and semantic association. As a consequence, the participants were able to create conceptual links, improved their long-term memory, and were able to more finely monitor their own cognitive and decision-making processes. This result cannot be imputed to the training of specific abilities that are tested within the IQ evaluation. Indeed, the MCNT approach does not involve any type of targeted cognitive training. The latter approach is controversial because some authors claim that it is effective in improving

TABLE 4 | Pairwise comparison pre vs. post-MCNT2.0 scores of WISC IV indices and VABS –II sub-scales.

	Scale	Variables/Score	MCNT$^{2.0}$ (N = 18)		Pairwise comparison T0 vs. T1	Cohen's d
			T0 Mean (*SD*)	T1 Mean (*SD*)	FDR-p-value	
Intellectual functioning	WISC-IV	VCI	82.11 (8.44)	86.44 (12.22)	0.29	0.40
		PRI	86.22 (9.33)	96.44 (15.90)	**0.040**	**0.74**
		WMI	78.33 (7.81)	82.33 (12.85)	0.22	0.36
		PSI	84.05 (11.42)	86.22 (13.92)	0.51	0.17
Adaptive functioning	VABS-II	FSQ	86.92 (10.18)	94.85 (12.13)	**0.040**	**0.70**
		Communication	81.08 (12.15)	90.38 (14.03)	**0.040**	**0.71**
		Daily living skills	90.38 (10.54)	98.92 (13.62)	**0.040**	**0.69**

MCNT, Movement Cognition and Narration of the emotions Treatment; WISC IV, Wechsler Intelligent Scale for Children IV; VCI, Verbal Comprehension Index; PRI, Perceptual Reasoning Index; WMI, Working Memory Index; PSI, Processing Speed Index; VABS-II, Vineland Adaptive Behavioral Scale II; FSQ, Full Scale Quotient; FDR, False Discovery Rate correction; Ns, not significant; Significant results are reported in bold font.

only the specific cognitive component that is trained. In this respect, some studies highlighted the difficulties in generalizing the trained ability to new learning context, from experimental setting to real life for example (53). In the MCNT, the Cognitive Lab focused on metacognitive strategies that are transversal to several cognitive competences, and more ecological. Our data are relevant in terms of clinical prognosis, because the improvement of global intellectual functioning can have a positive effect also on adaptive skills. Accordingly, a recent study (54) showed poorer emotion processing in adolescents with BIF when compared to healthy controls, and an inverse relationship between intellectual functioning and emotional awareness. The authors interpreted their results as evidence that borderline cognitive functioning affects mentalization processes and thus adaptive skills. For this reason, we consider it important, when working with children with BIF and ACE, to focus the treatment also on the intellectual abilities as defined above.

The main result of the present study is related to the effects of MCNT 2.0 on motor competencies in children with ACE and BIF. Data presented showed a significant increase in fine and gross motor functioning, as assessed with M-ABC and TGMD in children that underwent MCNT 2.0. This result relates to the only difference between the two versions of the MCNT. In the first version of the intervention, a Game Therapy approach was used, based on the use of the Wii and Xbox video game platforms, while the present version applied the Body-Minding, a method focused on both body movements with bimanual and interlimbic coordination exercises and body awareness. The choice of a Game therapy approach in the MCNT 1.0 was based on previous research that demonstrated its good efficacy in promoting engagement, motivation and motor competence in children (55–58) also in case of mental disability (59) and hand-eye coordination in adults (60). The present data, though, showed greater efficacy of the Body-Minding approach. A possible interpretation of this datum is that despite the "Game Therapy" approach is highly motivating and engaging for children, it only allows the choice of the type of game and its level of difficulty but not a finer tuning of the activities due to platform/game limitations. Conversely, the Body-Minding approach, even in a group setting, fostered an intervention on motor coordination and planning, and proprioceptive feeling, that was personalized and tailored on the basis of each child's strengths and difficulties. These data are in agreement with a previous study by Ferguson et al. (48) in which a Nintendo Wii Fit Training was compared to a Neuromotor Task Training (NTT), both carried out in a group setting to evaluate the impact on the performance of children with motor coordination problems. Children that participated in this study, similarly to our sample, attended mainstream primary schools and came from a low-income environment. Results showed that the NTT approach achieved broader and greater success than Wii training in motor proficiency, cardiorespiratory fitness and functional strength. On the contrary, children that underwent the Wii training, improved their anaerobic performance but their motor performance remained within the at-risk range. These data are relevant in light of the poor motor competences typically shown by children with BIF and ACE due to their difficulties in locomotor, object control, and fine motor skills (17, 61, 62).

The herein results on the motor performance of children with ACE and BIF are relevant because the motor difficulties observed in these children are strictly linked with the cognitive processes involving executive functions, such as inhibitory control and planning (63). Moreover, fine motor skills are highly related to the possibility to improve cognitive skills in pre-school children with intellectual disability and learning disorders (64). Indeed, the information arising from within the body, through visual, auditory, olfactive, tactile, and proprioceptive pathway need to be rapidly and efficiently processed and integrated to achieve a body awareness and to produce a goal-directed movement. Thus, intervening on the motor domain, can have an effect also on executive functions and vice-versa. Finally, driving attention on bodily sensation helps to feel, recognize, discriminate and regulate emotions that would otherwise remain confused, unwanted, and unexpressed. Indeed, in addition to cognitive and motor difficulties, children with ACE and BIF show emotional and behavioral difficulties associated (7, 13, 14, 65) with deficits in social competencies (12). Both versions of MCNT rehabilitation program provided specific interventions for emotion narration and recognition, the Emotion Lab, whose efficacy in improving social functioning and behavior was shown

by the results of the RCT study (33). The effect of treatment on social functioning was also confirmed in the MCNT 2.0 group. Moreover, significant increment in all indices of the VABS II scale was observed within this group. Unfortunately, the complete profile at the VABS II was available only for the MCNT 2.0 group and thus no comparison with the other group was possible. For this reason, it is not possible to rule out that these results are due to a test-retest effect. Finally, opposite to the results of the RCT, the CBCL score did not show significant changes in the MCNT 2.0 group. A possible interpretation of this result relates to differences in the specific difficulties and/or strengths of the children belonging to the two groups.

To summarize, children attending the MCNT 2.0 intervention improved their performances in all domains, especially in the motor domain whose improvement was more than one standard deviation. Notwithstanding, despite an increment in scores <1 standard deviation in the FSIQ and in the VABS II total score, these data are highly relevant from a clinical perspective for two main reasons. The first relates to the natural history of children with BIF and ACE that is not favorable, but is characterized by a high risk of long-term consequences. Thus, gaining a global improvement is potentially of great relevance. The second relates to the findings of a previous work from our group (33) in which results showed that working only on specific academic abilities produced improvement only in specific areas such as verbal memory and comprehension and did not generalize to adaptive and intellectual abilities. Moreover, this domain specific approach was associated with a paradoxical effect on behavior represented by a worsening on the CBCL scale. Consequently, our data support clinical decisions suggesting the importance of a multi domain therapeutic approach. To determine the long term clinical relevance of the MCNT intervention follow-up studies will be needed.

The present study is not free from limitations. This is a quasi-experimental study whose data from a non-randomized sample of subjects is compared to a secondary dataset from an RCT, according to the "Good Research Practices for Comparative Effectiveness Research" (34, 52, 66, 67). This approach potentially results in a selection bias of the children. To avoid this issue, we used the same inclusion criteria from the previous study and we included the first 20 consecutive children eligible for the study. Moreover, the children belonged to the same socioeconomic environment and from the same area of the children participating in the RCT. Moreover, the small group of participants prevent us from generalizing to the broad population of children with ACE and BIF. Finally, more follow-up is needed to evaluate the long-term effects of the treatment.

Despite these limitations, the herein data are relevant because of the importance to intervene in the developmental course of children experiencing ACE and with a BIF. This population is very vulnerable due to the high risk of school drop-out, poverty, and psychological problems in the adulthood (10, 13, 14, 68). Several data indicate that, in socio-economic disadvantaged contexts, children's IQ, together with the quality of the child/parent relationship, is one of the most important protective factor against maladaptive outcomes (36, 69). Indeed, one of the main goals of MCNT is to support resilience, through the multidomain and integrated approach that promotes the improvement of both the intellectual functioning and the emotional/relational competences of the children. The relational dimension is the real core of MCNT: everyone has an emotional and cognitive potential that can be enriched by the positive interaction with competent figures.

CONCLUSIONS

To conclude, our data support the starting hypothesis of the positive effects of our intensive, multidomain approach in children with ACE and BIF, and that a body awareness and interlimbic coordination approach has a greater effect in improving the motor functioning. The treatment of neurodevelopmental disorders is often expensive due to the necessity of a long-term care (70, 71). Is not clear how protective factors and resilience work in modifying the association between ACE and psychopathology. On the basis of our experience, we believe that a multimodal approach intervening on the three major domains, the cognitive, the motor, and the emotion, may positively impact the developmental processes and thus help prevent maladaptive outcomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available and will not be made publicly available because: the informed consent approved by the Ethics committee did not include any statement regarding the possibility to share the data. Requests to access the datasets should be directed to Gisella Baglio, gbaglio@dongnocchi.it.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committees of the Don Gnocchi Foundation and of the ASST S. Paolo and S. Carlo Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GB, MZ, FB, and VB conceived the study and wrote the manuscript. GB, VB, MC, and MZ executed the study. SD helped with statistical analyses. All authors contributed to refinement of the manuscript and approved the final content.

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Age- and Sex-Specific Standard Scores for the Reading the Mind in the Eyes Test

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The reliable, valid and economic assessment of social cognition is more relevant than ever in the field of clinical psychology. Theory of Mind is one of the most important socio-cognitive abilities but standardized assessment instruments for adults are rare. The Reading the Mind in the Eyes Test (RMET) is well-established and captures the ability to identify mental states from gaze. Here, we computed standard scores for the German version of the RMET derived from a large, community-dwelling sample of healthy adults (20–79 years). The standardization sample contains 966 healthy adult individuals of the population-based Leipzig Research Center for Civilization Diseases (LIFE) study. Before standardization, weighting factors were applied to match the current sample with distribution characteristics of the German population regarding age, sex, and education. RMET scores were translated into percentage ranks for men and women of five age groups (20–29, 30–39, 40–49, 50–59, 60+ years). Age-specific percentage ranks are provided for men and women. Independent of age, men present a larger variance in test scores compared to women. Within the specific age groups, women score higher and their scoring range is less variable. With increasing age, the scoring variance increases in both men and women. This is the first study providing age- and sex-specific RMET standard scores. Data was weighted to match German population characteristics, enabling the application of standard scores across German-speaking areas. Our results contribute to the standardized assessment of socio-cognitive abilities in clinical diagnostics.

Keywords: theory of mind, mindreading, Reading the Mind in the Eyes test, social cognition, neuropsychological testing, cognitive aging, dementia

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INTRODUCTION

Social cognition is one of the most complex and unresolved concepts in psychology and neurosciences. It encompasses numerous definitions (Happé et al., 2017) and includes many socio-cognitive abilities. The ability to identify mental states, intentions, desires, feelings and beliefs of self and others (Premack and Woodruff, 1978) is called Theory of Mind (ToM). It comprises the perception, processing and integration as well as the interpretation of social context information facilitating the prediction of another individual's future actions and the adaptation

of own behavioral responses. Thus, ToM contributes to successful social interactions. Contrary, impairment in ToM can compromise the initiation and maintenance of positive relationships. Among various psychiatric disorders and neurological diseases, brain injury, age-related brain changes, as well as developmental and neurodegenerative disorders (e.g., Gregory et al., 2002; Brune and Brune-Cohrs, 2006; Schroeter et al., 2010, 2014; Schroeter, 2012; Kynast et al., 2018; Vallat-Azouvi et al., 2018) have been characterized by ToM deficits. Further, the clinical relevance of ToM increased recently, when the Diagnostic Statistical Manual of Mental Disorders' 5th edition (DSM-5; American Psychiatric Association, 2013) explicitly defined social cognition as one key domain for neuropsychological assessment, especially in the context of acquired cognitive dysfunction, i.e., major and mild neurocognitive disorder (NCD; corresponding terms are dementia and its prestage, i.e., mild cognitive impairment, MCI).

Standardized psychological tests help to identify specific deficits (and resources) as they provide normative scores for the classification of individual abilities relative to a reference group (ideally) age-, sex- and education-matched. Performance deviations might indicate the need for subsequent in-depth testing to further evaluate and confirm the detected deficits as well as to classify potential impairment regarding activities of daily living. However, the systematic assessment of socio-cognitive abilities, particularly ToM, is problematic, as only a few instruments are available for adults and even fewer are standardized.

The *Reading the Mind in the Eyes Test* (RMET; Baron-Cohen et al., 1997, 2001) may be a potential candidate for standardized assessment of specific aspects of social cognition. It captures the ability to identify mental states based on the eyes of another person. The revised version (Baron-Cohen et al., 2001) includes 36 photographs of the eye region of 18 men and 18 women. Each item comes along with four response options. The term most appropriately describing the mental state of the pictured person shall be selected. The RMET requires the identification of complex mental states where social cues are limited (Baron-Cohen et al., 1997). The task addresses especially the identification of the relevant mental state of the stimulus (first stage of ToM attribution) rather than inferring the content of that mental state (second stage; see Baron-Cohen et al., 2001). It is supposed to capture predominantly "hot" or "affective" ToM, since it requires an understanding of another person's affective states or feelings (c.f. "cold" or "cognitive" ToM: a concept of cognitive states, beliefs, thoughts, or intentions; Henry et al., 2013; El Haj et al., 2015). This might be in line with a recent critical note on the validity of the RMET (Oakley et al., 2016), suggesting that the focus of the RMET is rather emotion recognition than ToM as compared to other assessment instruments using cartoons or short movies of social situations. Overall, studies show mixed results regarding the validity of the test (see Olderbak et al., 2015 for review of the psychometric properties; critical aspects regarding test construction can be found in Kynast and Schroeter, 2018). Besides these critical points, the RMET may still be a promising candidate for further clinical application since it has been shown to identify deficits in mental state recognition in psychopathology and neurodegenerative diseases such as behavioral variant frontotemporal dementia (Pardini et al., 2013; Schroeter et al., 2018), Alzheimer's disease and mild NCD (see Baglio et al., 2012), autism (Baron-Cohen et al., 1997, 2015), bipolar disorder (Bora et al., 2005) and small vessel disease (Kynast et al., 2018). The test is not characterized by a ceiling effect since the response rate of healthy individuals is typically below 100% (Pardini and Nichelli, 2009). Furthermore, it is one of the most frequently used tasks to investigate ToM in adulthood, published in more than 250 (Kirkland et al., 2013) or even 542 studies according to PubMed until 6th of April 2020 (term = reading + the + mind + in + the + eyes). Test performance is modulated by individual characteristics such as sex, and age, and the characteristics of the stimulus material itself (e.g., Baron-Cohen et al., 1997; Bailey et al., 2008; Pardini and Nichelli, 2009; Castelli et al., 2010; Cabinio et al., 2015; El Haj et al., 2015; Fischer et al., 2016; Kynast and Schroeter, 2018; Kynast et al., 2018, 2020). To our best knowledge, no standard scores are yet available in any language.

This study is the first to present age-standardized scores for the RMET derived from a large, population-based sample of healthy adults (Loeffler et al., 2015). We used the German version of the RMET (Bölte, 2005). Weighting factors were applied to match the current sample to the characteristic distribution of age, sex and education represented in the German population. RMET scores were transformed into percentile ranks that characterize the performance of an individual in relation to its reference group. The study aimed overall at yielding norms for neuropsychological characterization of adults and potentially identifying deviations due to disease.

METHODS

Study Cohort

This study was part of the adult study of the Leipzig Research Center for Civilization Diseases (LIFE). The LIFE adult study is a cross-sectional study investigating "prevalences, early onset markers, genetic predispositions, and the role of lifestyle factors of major civilization diseases" (Loeffler et al., 2015). The study was approved by the ethics committees of the University of Leipzig and was conducted in accordance with the latest version of the Declaration of Helsinki. Each subject provided written informed consent. All participants were randomly selected residents of the city of Leipzig (Saxony/Germany, population ∼600,000, Amt für Statistik und Wahlen Leipzig, 2020) aged 18–79 years. Participation was voluntary. The total sample comprised 10,000 individuals of whom 2,600 completed structural and functional brain magnetic resonance imaging (MRI). Adults aged 60–79 years completed an in-depth-assessment (neuropsychological testing, medical examinations, interviews on individual lifestyle conditions). Persons younger than 60 years completed a less extensive test battery. The complete protocol is described elsewhere (Loeffler et al., 2015).

For the current study, participants who fulfilled at least one of the following criteria were excluded from data analysis: (1) history of neurological or psychiatric disorder (i.e., dementia,

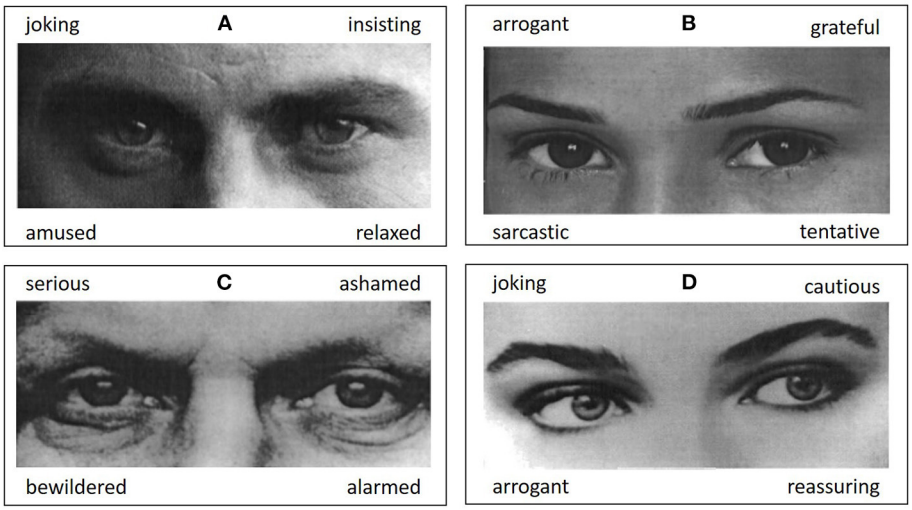

FIGURE 1 | Figure shows example items from the Reading the Mind in the Eyes Test (Baron-Cohen et al., 2001; Bölte, 2005). Out of four response options the most appropriate mental state term shall be selected. Correct responses are **(A)** insisting, **(B)** tentative, **(C)** serious, **(D)** cautious. Pictures are taken from Bölte (2005).

alcohol or substance abuse, schizophrenia, affective and anxiety disorders, eating disorders, autism), (2) intake of medication active on the central nervous system (opioids, hypnotics and sedatives, anti-parkinsonian drugs, anxiolytics, antipsychotics, anti-epileptic drugs), (3) depression score >20 on the Center of Epidemiologic Studies Depression Scale (Radloff, 1977), (4) history of stroke, brain injury or tumor, (5) significant white matter hyperintensities (Fazekas stages 2 and 3) on T2-weighted fluid attenuated inversion recovery (FLAIR) MRI scans (Fazekas et al., 1987). The latter has been selected as a specific exclusion criterion since age-related changes in the brain's white matter structure are associated with cognitive impairment. Especially structural alterations as severe as Fazekas stages 2 and 3 have been associated with significant cognitive deficits in attention, memory, and most crucially, social cognition compared to individuals without white matter hyperintensities (Kynast et al., 2018).

The final standardization sample included 966 adults (48.6% male) aged 20–79 years (mean, $M = 50.7$; standard deviation, $SD = 16.2$). Demographic characteristics, self-report on medical history and medication intake, as well as brain MRI was available for the whole sample. Note that all individuals had normal or corrected to normal vision.

Test and Procedure

The ability to identify mental states from gaze was assessed with the German version of the revised RMET (Baron-Cohen et al., 2001; Bölte, 2005). The test contains 36 black-and-white photographs of the eye region of either a man or a woman. Each item is presented with four response options of which the word best describing the pictured mental state shall be selected (see **Figure 1** for examples). Computerized assessment included standard instructions and a test item (Bölte, 2005). If necessary, trained study assistants provided help with the handling of the hardware for response selection. Assessment was self-paced and needed approximately 10-15 min for completion. Prior to analysis, data was carefully checked for plausibility, i.e., possible biases in response profiles as well as accuracy rates below chance level (<25%), indicating potential nonconformity with the test instructions. No individual of the final analysis sample scored below chance level nor did study documentation indicate irregularities regarding task completion.

Statistical Analysis

Dataset Preparation

Firstly, the analysis sample was stratified into sex (male/female), age [20–29 years, 30–39 years, 40–49 years, 50–59 years, and 60 years and older (60+)] and education (≤10 years of school; >10 years of school) groups providing a basic data structure for the calculation of RMET standard scores. Since these standard scores should be applicable for German-speaking subjects, it was necessary that the distribution characteristics of the study sample match the German population regarding the above-mentioned characteristics. As this was not the case *per se*, weighting factors were computed based on German population data published in the "Mikrozensus 2014" study (Statistisches Bundesamt Wiesbaden, 2018). The weighting factors were derived by aligning the number of individuals of the Mikrozensus 2014 age-sex-education subsamples with the number of individuals of the corresponding subsamples of the current study. Weighting factors were then multiplied group-wise with the number of individuals of the study dataset resulting in an overlap with the German population characteristics. See **Table 1** for a detailed overview about weighting factor extraction and application to the study dataset.

RMET Standard Score Computation

This computation is performed on the aligned dataset (i.e., where weighting factors were applied to match the German population according to age, sex, education). Age-specific frequency distributions of correct responses in the RMET for

TABLE 1 | Number of participants per group in the German reference population (Mikrozensus 2014; $N = 63{,}292$), the LIFE adult study ($N = 966$), and the weighted analyses sample (LIFE*, $N = 966$).

Sex	Education	N Mikrozensus (%)	*N* LIFE (%)	Weighting factor	*N* LIFE*
A. Age group: 20–29 years					
Male	≤10 y	2437 (3.9)	12 (1.2)	3.1	37
	>10 y	2156 (3.4)	30 (3.1)	1.1	33
Female	≤10 y	2043 (3.2)	3 (0.3)	10.4	31
	>10 y	2388 (3.8)	15 (1.6)	2.4	36
Total	≤10 y	4480 (7.1)	15 (1.6)	4.6	68
	>10 y	4544 (7.2)	45 (4.7)	1.5	69
B. Age group: 30–39 years					
Male	≤10 y	2711 (4.3)	20 (2.1)	2.1	41
	>10 y	2038 (3.2)	38 (4)	0.8	31
Female	≤10 y	2518 (4)	9 (0.9)	4.3	38
	>10 y	2186 (3.5)	24 (2.5)	1.4	33
Total	≤10 y	5229 (8.3)	29 (3)	2.8	80
	>10 y	4224 (6.7)	62 (6.4)	1	64
C. Age group: 40–49 years					
Male	≤10 y	3809 (6)	58 (6)	1	58
	>10 y	2076 (3.3)	27 (2.8)	1.2	32
Female	≤10 y	3800 (6)	44 (4.6)	1.3	58
	>10 y	1908 (3)	20 (2.1)	1.5	29
Total	≤10 y	7609 (12)	102 (10.6)	1.1	116
	>10 y	3984 (6.3)	47 (4.9)	1.3	61
D. Age group: 50–59 years					
Male	≤10 y	4075 (6.4)	28 (2.9)	2.2	62
	>10 y	1800 (2.8)	18 (1.9)	1.5	27
Female	≤10 y	4376 (6.9)	27 (2.8)	2.5	67
	>10 y	1556 (2.5)	9 (0.9)	2.6	24
Total	≤10 y	8451(13.4)	55 (5.7)	2.3	129
	>10 y	3356 (5.3)	27 (2.8)	1.9	51
E. Age group: ≥60 years					
Male	≤10 y	7281 (11.5)	201 (20.8)	0.6	111
	>10 y	2349 (3.7)	138 (14.3)	0.3	36
Female	≤10 y	10351 (16.4)	176 (18.2)	0.9	158
	>10 y	1434 (2.3)	69 (7.1)	0.3	22
Total	≤10 y	17632 (27.9)	377 (39)	0.7	269
	>10 y	3783 (6)	207 (21.4)	0.3	58

Panels A–E show group-specific absolute number of participants per group in the German reference population (Mikrozensus 2014) and the LIFE adult study. Relative numbers are presented in parenthesis. Weighting factors are calculated as: relative number (Mikrozensus)/relative number (LIFE). In a last step, weighting factors are multiplied with the original number of participants from the LIFE adult study resulting in the final distribution of the current study's analysis sample (N LIFE).*

men and women have been used. For each frequency distribution, percentile ranks were computed as $PR_v = \frac{freq_{cum}(x_v)}{N} * 100$ (Moosbrugger and Kelava, 2012, p. 176 et seqq.). The term $freq_{cum}(x_v)$ defines the accumulated frequencies up to (and including) the score of interest (i.e., RMET accuracy score); N defines the number of participants within the (sub-) sample. The percentage rank informs about an individual's accuracy in the RMET within the reference group. It is defined as the relative number (percentage) of scores within the frequency distribution equal to or lower than it. Percentile ranks were computed separately for men and women of five age groups (20–29, 30–39, 40–49, 50–59, 60+).

RESULTS

The sample comprises 966 adults (469 male) aged 20–79 ($M = 50.7$, $SD = 16.2$; see **Table 2**). Descriptive results suggest a trend toward an inverse relation between RMET accuracy rates and age. Also, females scored slightly higher than males on a descriptive level. The sex-specific RMET raw score distribution across the sample is additionally presented in **Figure 2**. Although women scored on the top level (max accuracy score = 32), both men and women presented identical accuracy spans (20 scores).

Persons with higher education presented better RMET performance compared to persons with <10 years of school

TABLE 2 | Sociodemographic characteristics of the standardization sample (N = 966) and corresponding RMET scores (M, SD).

		N	%	RMET total correct *M* (*SD*)
Age (years)	$M = 50.7$	966	100	
	$SD = 16.2$			
	range: 20–79			
Age groups	20–29	138	14.2	26.0 (3.2)
	30–39	144	14.9	24.7 (3.2)
	40–49	177	18.3	24.1 (3.3)
	50–59	180	18.7	23.4 (3.2)
	60+	327	33.8	22.4 (3.8)
Sex	Male	469	48.6	23.4 (3.6)
	Female	497	51.4	24.1 (3.7)
Education	≤10 years	662	68.6	23.3 (3.7)
	>10 years	304	31.4	24.8 (3.4)

N, sample size; M, mean; RMET, Reading the Mind in the Eyes Test; SD, standard deviation.

(**Table 2**). An one-way ANOVA predicting RMET accuracy from age-group (5), sex (2) and education (2) was computed [$F_{(17;945)} = 9.992$, $p < 0.001$; R^2=13.6]. Main effects of the factors age [$F_{(4;945)} = 24.452$, $p < 0.001$), sex [$F_{(1;945)} = 8.727$, $p < 0.001$], and education [$F_{(1;945)} = 6.571$, $p < 0.001$) were found.

Normative scores are shown in **Table 3**. Here, ranked raw test scores were monotonously transformed into percentile ranks within a specific age and sex group (see again **Table 1** for information on weighting factor extraction and application to the study dataset). The maximum scores within an age group are comparable between men and women. Regarding age, older age groups present a wider scoring range compared to younger age groups. For older men/women, lower accuracy rates may therefore be defined as age-appropriate, while an identical score may be classified as "below average" in a younger individual. For instance, a number of 20 correctly identified items corresponds to a percentile rank of 12 in a man younger than 30 (below average), while performance is on average for a man in his 60's with an identical score. Note, that for certain raw scores no corresponding percentage rank could be calculated.

DISCUSSION

In this study, we computed age- and sex-standardized scores for the German version of the RMET based on a population-based sample including 966 healthy adults. Scores were weighted to match the demographic characteristics of the German population. This is the first study publishing standard scores for the frequently used RMET. The RMET assesses a specific aspect of ToM, i.e., the ability to identify complex mental states from gaze. Based on the guidelines for the evaluation of cognitive performance in the framework of NCD introduced by the DSM-5 (American Psychiatric Association, 2013), we provide age- and sex-specific standard scores improving the reliable distinction between typical and atypical test performance for a potential application in the clinical diagnostics of social cognitive abilities.

Notably, this study goes beyond clinical aspects of socio-cognitive impairment, as it addresses the ability to identify mental states from gaze in healthy adults across the adult age range. With this, it is linked to the concept of lifespan development (Baltes, 1987). Age may affect social cognitive abilities differently in men and women, but recent findings regarding the RMET are contradictory (e.g., Kirkland et al., 2013). Our results suggest slight performance differences between men and women, justifying the computation of specific standard scores. In both men and women, performance is reduced with older age.

Besides the remarkably large number of individuals used for standardization and the wide age-span covered, data was weighted according to age, sex, and education status to match the population characteristics of Germany. With this, standard scores are not limited to the current sample, but may be generally applied across German-speaking areas. Notably, rank transformation does not require normal distribution, but the shape of the distribution function informs about the group-specific frequency of RMET scores (cf. Moosbrugger and Kelava, 2012). This may be helpful for the interpretation of individual performance in relation to the standardization sample. Importantly, sections within the frequency distribution where scores are denser may lead to an overestimation of performance differences, while performance differences in less dense sections may be underestimated.

By addressing these points, our study contributes essentially to a systematic assessment of socio-cognitive abilities, which is an important aspect in clinical diagnostics of psychiatric, neurological, and neurodegenerative diseases associated with ToM impairment. Moreover, defining the range of extraordinary test performance enables to identify persons with distinct social cognitive abilities, which may serve as an important personal resource and cognitive reserve deferring cognitive decline.

Limitations and Implications for Application in Diagnostic Settings

Although this study was carefully conducted, some limitations need to be considered. Firstly, the stratification of the current sample according to sex, age, and education as a basic structure for standard score computation may seem arbitrary. However, it is common sense in the field of neuropsychology that basic demographic factors potentially impact cognitive performance. The ANOVA results additionally justify this approach. Additionally, it enables comparability with other studies and facilitates application in clinical settings. The binary coding of sex/gender information may be regarded conventional as it neglects persons with differing sexual identity and personal self-concept potentially influencing socio-cognitive functioning (e.g., Kung, 2020). Thus, standard scores provided here may not be applicable in these cases. In future, it may be helpful to consider concepts beyond the classical sex/gender dimorphism picturing sexual diversity in populations. Of note, information on sex/gender was obtained directly from the resident's registration office of the city of Leipzig, and, additionally, via self-report. All participants showed agreement

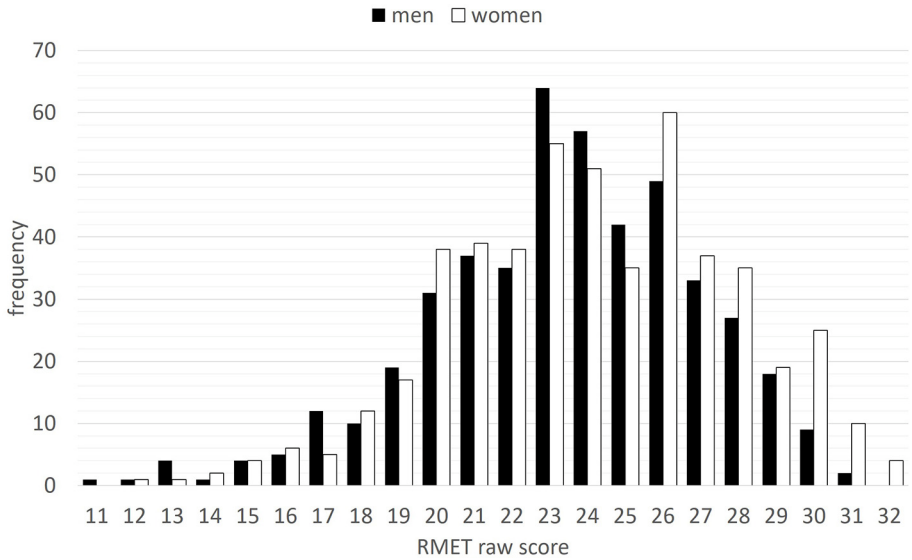

FIGURE 2 | Sex-specific frequency distribution of correct responses in the *Reading the Mind in the Eyes Test (RMET)* across the standardization sample ($N = 966$).

on both factors (see also Kynast et al., 2020). Furthermore, other factors such as environment, culture and experience may drive (social) cognitive performance beyond sex/gender (see Jäncke, 2018 for review; but also Dotson and Duarte, 2020 for the importance of sex/gender in neuroscience). Secondly, education was dichotomized based on years of formal education and graduation diploma for practicability reasons. Yet, tertiary education or further professional training was not considered for standardization although it possibly modulates RMET performance. This effect on mental state attribution remains subject to future research. Thirdly, numbers of subjects differ in the several groups. Here, the relatively large age group 60+ spans two decades of adults instead of being separated into one group for each decade (60–69, 70–79). This was done to provide an appropriate number of elderly individuals with higher education (cf. **Table 1**) for standardization, since formal scholastic education of more than 10 years was unusual among persons growing up from between the late 1930s and early 1950s in Germany, which can be regarded as a cohort effect. Although a broad definition of this age group may thus be justified and the application of weighting factors ensures matching distribution characteristics with the German population, it must be considered that the individual test performance of a person with advanced age may be characterized less precisely under these conditions. The original study distribution did not exactly match the characteristic proportions regarding age, sex and education in the German population, which is a limitation of the study. We tried to overcome this issue by the application of weighting factors. We based this on the latest available distribution data (Statistisches Bundesamt Wiesbaden, 2018). With more than 950 individuals, the sample size can be regarded appropriate for standardization. Yet, the subsamples used for the computation of sex-specific standard scores contain <100 individuals per group and it must be noted that in those groups not all percentage ranks may correspond to distinct RMET raw scores. Hence, our data corresponds with previous studies since again, the test does not have a ceiling effect (Pardini and Nichelli, 2009), supporting its eligibility for clinical application.

However, it must be critically addressed that the standard scores provided here are not sufficient for the diagnosis of psychiatric disorders, neurological or neurodegenerative diseases but rather indicate difficulties in a specific aspect of ToM, i.e., the ability to identify mental states from gaze. Deficits obtained in this test must be verified by subsequent, in-depth diagnostic procedures. Furthermore, RMET performance may be modulated by other individual factors that have not been considered here. For instance, it has been shown that RMET performance is related to verbal intelligence, but also other cognitive abilities (Ahmed and Miller, 2011; Peterson and Miller, 2012; Baker et al., 2014; Cabinio et al., 2015; Kynast et al., 2018, 2020) that possibly enhance or decrease mindreading accuracy. Moreover, test performance may be influenced by the RMET's stimulus characteristics (Kynast and Schroeter, 2018). Thus, these factors should be additionally assessed and considered in diagnostic settings.

Overall, the RMET, with all its strengths and limitations, can thus be a useful assessment instrument indicating socio-cognitive deficits in specific psychiatric disorders and neurological diseases. In a more resource-oriented view this test may also be used to detect individual strengths in this cognitive domain that might improve the use of therapeutic interventions or be used in professional settings (e.g., identify applicants with extraordinary skills in specific sectors such as health care or elderly care).

TABLE 3 | Age- and sex-specific percentile ranks corresponding to number of correct responses in the *Reading the Mind in the Eyes Test (RMET)*.

Sex			**Men**					**Women**		
Age group (years)	20–29	30–39	40–49	50–59	60+	20–29	30–39	40–49	50–59	60+
N	70	72	90	90	147	68	72	87	91	180
RMET raw score					**Percentile rank**					
≥32						98				
31						96	98			
30	98	98			99	89			97	99
29	92	94	98		98	70	96	92		97
28	84	91	94	95	97	66	82	87	94	95
27	72	88	85	91	92	47	74	80		92
26	63	77	78	82	89	40	56	74	88	85
25	46	58	72	73	82	33	47	51	69	79
24	41	50	61	63	72	29		42	63	70
23	34	37	47	53	58	19	41	32	47	58
22	23	24	32		49	15		21	41	46
21		15	25	30	38		19	16	33	36
20	12	9	20	15	29		15	10	19	26
19		6	12	12	21		5		11	17
18			10	6	15			3	5	13
17		1	8	4	11			1		9
16			3		8					7
15			1	2	6					4
14					5					2
13					4					1
≤12					1					

N, sample size.

Conclusion

In conclusion, this is the first study providing standard scores for the ability to identify mental states from gaze. They may be used for the detection of socio-cognitive deficits in clinical practice, e.g., in the context of dementia, other neurological diseases and psychiatric disorders, or the assessment of socio-cognitive resources that may be strengthened by prevention or rehabilitation strategies. This large sample of healthy adults was weighted to match the distribution characteristics regarding age, sex, and education of the German population, enabling the application of RMET standard scores to German-speaking areas.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data can be requested upon reasonable request. Requests to access these datasets should be directed to Matthias L. Schroeter, schroet@cbs.mpg.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee University of Leipzig. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JK planned and conducted statistical analyses, wrote the first draft of the manuscript including creation of the figures, and modified all subsequent drafts. MP was responsible for data quality management and contributed substantially to all drafts of the manuscript. EQ planned the study, assessed and processed data, and substantially contributed to all drafts of the paper. AH contributed substantially to the interpretation of the results and to the manuscript. AV designed the study, interpreted results, and reviewed the final draft of the manuscript. MS designed the study, supervised data acquisition and analyses, substantially contributed to the interpretation of the results, and made substantial modifications to all drafts of the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

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Evolution of Apathy in Early Parkinson's Disease: A 4-Years Prospective Cohort Study

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Objective: We investigated the prevalence, evolution, associated factors, and risk factors of apathy in a cohort of patients with early-stage Parkinson's disease (PD), who underwent a 4-years prospective follow-up.

Methods: This study included 188 patients with PD (baseline disease duration <3 years) who underwent an annual evaluation using the Lille Apathy Rating Scale (LARS). Based on the cut-off value of −21 observed on the LARS, patients were categorized as PD with and without apathy. The generalized estimating equations (GEE) model was utilized to determine the factors associated with apathy, and the Cox proportional-hazards regression model was used to determine the predictors of apathy.

Results: Apathy increased from a baseline rate of 18.6–28.8% after 4 years; notably, this rate was not persistent across patients' visits. The LARS score was independently associated with the male sex (B 8.131, $p = 0.009$), low Frontal Assessment Battery (FAB) scores (B 0.567, $p = 0.011$), low attention scores on the Montreal Cognitive Assessment (MOCA) test (B 0.217, $p = 0.026$), high Hamilton Depression Rating Scale (HDRS) scores (B 1.362, $p < 0.001$), high Unified Parkinson's Disease Rating Scale (UPDRS) part III scores (B 1.147, $p < 0.001$), and prolonged follow-up time (B 1.785, $p = 0.048$). A high HDRS score was the only predictor of apathy in PD [hazard ratio (HR) 1.043, $p = 0.026$].

Conclusions: The risk of apathy is higher in men with progressive PD accompanied by disease-specific motor and non-motor symptoms. Depression during early-stage PD is a primary risk factor for apathy in PD.

Keywords: Parkinson's disease, apathy, depression, executive function, cohort study

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INTRODUCTION

Apathy, a common neuropsychiatric symptom in patients with Parkinson's disease (PD), can occur both in the early and advanced stages of PD and may even precede the motor symptoms of the disease (Pagonabarraga et al., 2015). Apathy is a non-motor symptom that refers to a state of reduced motivation that manifests as diminished goal-directed behaviors, reduced interests, or emotional features that cannot be attributed to altered levels of consciousness, cognitive impairment, or emotional distress (Marin, 1991). Reportedly, apathy is associated with older age, cognitive impairment, depression, and more severely disabling disease in patients with PD

(Pagonabarraga et al., 2015), which can negatively affect the quality of life of patients (Gerritsen et al., 2005) and increase the burden of the caregiver (Martinez-Martin et al., 2015).

Most previous studies on apathy in PD have used cross-sectional designs. Although apathy is commonly observed in patients with PD, its prevalence varies significantly (ranging from 16.5 to 60%; Den Brok et al., 2015). To date, few prospective studies with a repeated measures design have focused on apathy in PD. Most studies have included a short follow-up period or a small sample size (Pedersen et al., 2009; Santangelo et al., 2015b; Wee et al., 2016). Therefore, limited data are available regarding the long-term evolution and trajectory of apathy in patients with early-stage PD; further research is warranted to gain a deeper understanding of the associated and predicted factors of apathy in patients with PD. This information would benefit clinicians in real-world practice because apathy is often associated with poor prognosis (Starkstein et al., 2006) and poor response to treatment (Mega et al., 1999).

We prospectively investigated patients with early-stage PD, who underwent a 4-years follow-up to determine the prevalence, evolution, associated factors, and risk factors of apathy in patients with PD.

METHODS

Study Design and Population

This study is a part of an ongoing long-term prospective longitudinal cohort study performed at the Department of Neurology, West China Hospital, Sichuan University, to investigate the progression and prognosis of PD in Chinese patients ($n = 302$). This project initiated in February 2014 and aimed to recruit patients with early PD (disease duration <3 years) to a follow-up of at least 10 years. PD was diagnosed based on the United Kingdom Parkinson's Disease Society Brain Bank's clinical diagnostic criteria for PD (Hughes et al., 1992). We excluded patients with dementia, patients with motor complications (including motor fluctuation and dyskinesia), and patients with Hoehn and Yahr (H&Y) stage ≥3 at baseline.

All patients underwent standardized examinations and regular assessments by trained neurologists in our movement disorder center annually. In the present study, the data analysis was performed based on the assessments at baseline, as well as a 1-, 2-, 3-, and 4-years follow-up. Of the 302 patients recruited, 114 were excluded owing to lack of data regarding the assessment of baseline apathy ($n = 109$) or missing data from other assessments at follow-up visits ($n = 5$); therefore, only 188 patients were eligible for inclusion in the study. All included participants completed the baseline and 1-year follow-up visits. The number of patients who lost contact or withdrew informed consent during 2-, 3-, and 4-years follow-up were 5, 63, and 81, respectively. One patient died between 2- and 3-years follow-up and 5 patients died between 3- and 4-years follow-up. In addition, 49 patients had not yet reached the time to finish the 4-years follow-up visit before we carried out the data analysis. Therefore, the number of patients we included at baseline, 1-, 2-, 3-, and 4-years were 188, 188, 183, 124, and 52, respectively.

The study was approved by the Ethics Committee of West China Hospital, Sichuan University, and written informed consent was obtained from all patients.

Evaluation Protocol

Baseline demographic data and clinical characteristics, including sex, age, age of disease onset, disease duration, and years of schooling, were collected, and the therapeutic regimen was recorded at each visit. Antiparkinsonian medications of patients were converted into the total levodopa equivalent daily doses (LEDD) based on a previous report (Tomlinson et al., 2010). All patients with PD underwent baseline and periodic neurological evaluation during the follow-up. The severity of motor symptoms associated with PD was evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS) part III (Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease, 2003) and the H&Y stage (Hoehn and Yahr, 1967). The Hamilton Depression Rating Scale (HDRS) containing 24 items (Moberg et al., 2001) was used to evaluate depression, and the Hamilton Anxiety Rating Scale (HADS; Hamilton, 1959) was used to evaluate anxiety. The executive function was evaluated using the Frontal Assessment Battery (FAB; Dubois et al., 2000). The Montreal Cognitive Assessment (MOCA) screening tool was used for global cognitive function evaluation (Nasreddine et al., 2005). The MOCA contains the following seven subdomains: visuospatial/executive ability, naming, attention, language, abstraction, memory, and orientation.

Apathy Evaluation

Apathy was evaluated annually using the Lille Apathy Rating Scale (LARS) (Leentjens et al., 2008), a validated scale to assess apathy in PD. The total LARS score ranges between −36 and +36, with cut-off values for non-apathy, slight apathy, moderate apathy, and severe apathy being (−36 to −22), (−21 to −17), (−16 to −10), and (−9 to +36), respectively. The prevalence of apathy observed at each visit was calculated based on the percentage of patients with a LARS score of ≥-21 (Leentjens et al., 2008).

Statistical Analyses

All statistical analyses were performed using SPSS software, version 22.0, or R software, version 4.0.2. All statistical tests were two-tailed, and the values of $p < 0.05$ were considered statistically significant. Continuous variables are represented as means and SD, and categorical variables as counts and percentages. The chi-square test, the Fisher's exact test, and the Student's *t*-test were used for an intergroup comparison of the clinical variables.

Population-averaged regression models using generalized estimating equations (GEE) with multiple linear regression analysis were used to determine the factors associated with the severity of apathy. The models were based on all patients in the cohort, included all consecutive examinations over the follow-up period, and allowed for a correlation between repeated measurements obtained in the same patients. An exchangeable

TABLE 1 | Demographic and clinical features of PD patients.

	Baseline	1-year	2-years	3-years	4-years
Number of samples	188	188	183	124	52
Age, years, mean (SD)	58.1 (10.7)	59.3 (10.7)	60.7 (10.7)	61.2 (11.1)	63.6 (10.4)
Disease duration, mean (SD)	1.5 (0.8)	2.7 (1.0)	4.1 (1.1)	5.2 (1.2)	6.0 (1.1)
Male sex, *n* (%)	95 (50.5)	95 (50.5)	93 (50.8)	62 (50.0)	31 (59.6)
Education, mean (SD)	10.7 (4.1)	10.7 (4.1)	10.7 (4.1)	10.7 (4.2)	10.6 (4.0)
LEDD, mg/day, mean (SD)	152.5 (188.1)	329.9 (174.9)	430.8 (195.7)	537.5 (211.6)	529.8 (210.7)
Use of levodopa, *n* (%)	74 (39.4)	128 (68.1)	148 (80.9)	113 (91.1)	50 (96.2)
Use of dopamine agonist, *n* (%)	46 (24.5)	124 (66.0)	152 (83.1)	110 (88.7)	47 (90.4)
Use of anti-depressant, *n* (%)	8 (4.3)	6 (3.2)	17 (9.3)	12 (9.7)	4 (7.7)
Use of AChE-I, *n* (%)	0	0	0	1 (0.8)	1 (1.9)
FAB score, mean (SD)	16.4 (1.9)	16.3 (2.2)	16.3 (2.0)	16.4 (1.8)	16.3 (2.1)
MOCA score, mean (SD)	25.5 (3.5)	26.0 (3.5)	25.8 (3.5)	25.8 (3.6)	25.8 (3.7)
HDRS score, mean (SD)	7.8 (7.2)	7.2 (6.4)	7.3 (6.4)	7.6 (5.9)	8.0 (6.1)
HARS score, mean (SD)	5.6 (5.5)	6.2 (6.0)	6.0 (5.3)	6.2 (5.3)	7.0 (5.7)
UPDRS III score, mean (SD)	23.3 (10.6)	25.4(10.6)	27.5 (10.7)	29.1 (11.9)	31.2 (10.1)
H&Y, mean (SD)	1.9 (0.4)	2.0 (1.8)	2.1 (0.5)	2.2 (0.5)	2.2 (0.4)
LARS, mean (SD)	−27.0 (10.4)	−27.3 (7.6)	−26.1 (9.2)	−25.5 (8.8)	−25.0 (9.4)

PD, Parkinson's disease; LEDD, levodopa equivalent daily dose; AChE-I, acetylcholinesterase inhibitor; FAB, frontal assessment battery; MOCA, Montreal Cognitive Assessment; HDRS, Hamilton Depression Rating Scale; HARS, Hamilton Anxiety Rating Scale; UPDRS, Unified Parkinson's Disease Rating Scale; LARS, Lille Apathy Rating Scale.

working correlation structure was selected. The dependent variable, the LARS score, was used as a continuous variable in the model. The independent variables included the following repeated measures: age, sex, disease duration, education level, the LEDD, the administration of levodopa, the administration of dopamine agonists, the administration of antidepressants, the UPDRS part III score, the H&Y stage, the FAB score, the total MOCA score along with its subdomain scores, the HDRS score, the HARS score, and the follow-up time in years. The GEE analysis was first performed using only a single covariate at a time (unadjusted model) and was subsequently performed using all covariates that showed the values of $p < 0.1$ or were possibly associated with apathy (adjusted model).

The univariate and multivariate Cox proportional-hazards regression models were used to determine the predictors of apathy in PD. These models were used for the evaluation of patients who reported the absence of baseline apathy. The clinical outcome was defined as an onset of apathy (both persistent and non-persistent) observed during the follow-up. The univariate analysis (unadjusted model) included only a single covariate at a time. The multivariate analysis (adjusted model) included the following variables: sex, the FAB score, the HDRS score, and the UPDRS III score based on the values of $p < 0.1$ or a probable association with apathy based on clinical judgment. The Schoenfeld individual test was used to determine the proportional hazard assumption, where a value of $p > 0.05$ indicated that the data met the criteria.

Data Availability

Anonymized data can be obtained upon request from qualified investigators for the purposes of replicating procedures and results.

RESULTS

Baseline and Follow-Up Data

No statistically significant differences were observed in the baseline characteristics between patients who were included in the current study ($n = 188$) and those who did not ($n = 114$; **Supplementary Table 1**). The demographic and clinical features of patients with PD included in this study are listed in **Table 1**. We included 188 patients with PD at baseline (50.5% men). The mean patient age at baseline was 58.1 (SD 10.7) years, with mean disease duration of 1.5 (SD 0.8) years. The LARS score increased from −27 (SD 10.4) at baseline to −25 (SD 9.4) after 4 years. Baseline antiparkinsonian therapy was administered to 90 patients (47.9%), and this figure increased to 100% after 4 years, with a mean increase in the LEDD from 152.5 mg/day (SD 188.1) to 529.8 mg/day (SD 210.7).

Prevalence and Evolution of Apathy

Figure 1 shows the observed point prevalence of apathy in patients with PD. Of the 188 patients, 35 (18.6%) had apathy at baseline. During the follow-up, we observed that the 1-, 2-, 3-, and 4-years prevalence rates increased to 20.2% (38/188), 25.7% (47/183), 26.6% (33/124), and 28.8% (15/52), respectively.

We observed that in most patients with PD, apathy was not persistent across visits during the 4-years study period (**Figure 2**). Nine patients with PD showed persistent apathy after 2 years, whereas only three patients and two patients showed persistent apathy after 3 and 4 years, respectively.

A total of 52 patients completed the 4-years follow-up. No significant difference was observed in the baseline characteristics between patients with and without complete 4-years follow-up (**Supplementary Table 2**).

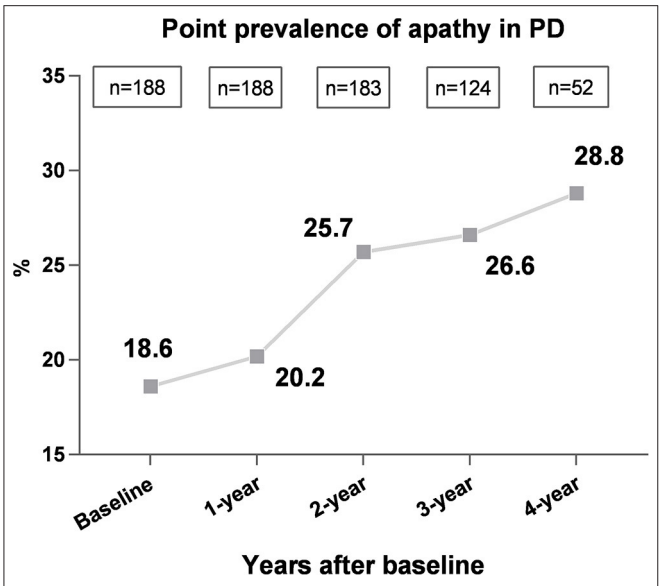

FIGURE 1 | Frequency of apathy over time in patients with PD. The prevalence of apathy in patients with PD increased with disease duration over time, which increased from 18.6% at baseline to 28.8% after 4 years.

Factors Associated With Apathy in Patients With PD Over Time

At baseline, patients with apathy had a significantly lower score in the FAB ($p = 0.005$) and higher scores in the HDRS ($p < 0.001$), HARS ($p = 0.007$), and UPDRS III ($p = 0.004$; **Supplementary Table 3**).

Table 2 shows the factors associated with the LARS score in PD over time. The GEE analyses indicated that the LARS score was independently associated with the male sex [B 8.131, 95% confidence interval (CI) 1.673–39.521, $p = 0.009$], low FAB scores (B 0.567, 95% CI 0.366–0.879, $p = 0.011$), low attention subscores on the MOCA screening test (B 0.217, 95% CI 0.056–0.833, $p = 0.026$), high HDRS scores (B 1.362, 95% CI 1.176–1.577, $p < 0.001$), high UPDRS part III scores (B 1.147, 95% CI 1.064–1.236, $p < 0.001$), and prolonged follow-up time (B 1.785, 95% CI 1.005–3.168, $p = 0.048$; adjusted model).

To explore the effect of drugs on the conversion of apathy, we further compared the change in LEDD between patients with and without persistent apathy in PD. At four stages (baseline to 1-year, 1- to 2-years, 2- to 3-years, and 3- to 4-years), the change in LEDD was not significantly different (**Supplementary Table 4**).

Predictors of Apathy in Patients With PD

Table 3 shows the risk factors for apathy in PD. The Cox proportional-hazards regression model indicated that a higher HDRS score was the only predictor of apathy in PD [hazard ratio (HR) 1.043, 95% CI 1.005–1.081, $p = 0.026$; adjusted model]. Male sex, baseline FAB score, and baseline UPDRS part III scores were not associated with the development of apathy in PD. The Schoenfeld individual test indicated that the Schoenfeld residuals were not significantly associated with time ($p = 0.094$), suggesting that the Cox model met the proportional hazard assumption (**Supplementary Figure 1**).

DISCUSSION

In this prospective longitudinal study, we observed an increased prevalence of apathy over time (from 18.6 to 28.8%) in patients with PD; however, apathy was not persistent. We also observed that the severity of apathy was associated with the male sex, the severity of motor symptoms, attention deficits, executive dysfunction, and depression in patients with early PD. Interestingly, the severity of depression was the only predictor for the onset of apathy in patients with PD. Our results highlight that apathy is an early, common, and non-persistent non-motor symptom in patients with early PD and may, therefore, have implications for clinical management.

Although numerous studies have investigated the prevalence of and factors associated with apathy in patients with PD, most of these were cross-sectional studies. Reportedly, the prevalence of apathy ranges between 14 and 40% in patients with early drug-naïve PD (Aarsland et al., 2009; Pedersen et al., 2010; Dujardin et al., 2014; Santangelo et al., 2015b; Liu et al., 2017), which could perhaps be attributed to the differences in study designs, particularly the differences in the definition of apathy, which was assessed by either the LARS, the neuropsychiatric inventory, or a self-reported version of the Apathy Evaluation Scale.

In the current study, the prevalence of apathy increased with disease progression; however, the symptom was not persistent. At the 4-years follow-up, we observed a 1.5-fold increase in the prevalence of apathy among patients with PD, although the overall prevalence was relatively low during the early stage (<30%). Our findings indicate that apathy is one of several major neuropsychiatric symptoms experienced by patients with early PD. The non-persistent property of apathy increases the difficulty to predict its development. The limited sample of patients with persistent apathy in our cohort also contributes to the impossibility to analyze the predictors of persistent apathy in the current study. Further, larger sample studies with the stratified method are needed to clarify the determinants of apathy. Moreover, the association observed between a high LARS score and a prolonged follow-up time in years indicated that the severity of apathy was likely to increase with disease duration, suggesting that the severity of apathy increases with disease progression in patients with PD.

In our study, apathy was more severe among patients with more severe motor disability; this finding is consistent with the results reported by two previous cross-sectional studies (Pedersen et al., 2010; Dujardin et al., 2014) and also supports our prior findings (Liu et al., 2017). These results imply that the dysfunction of the dopaminergic system is a likely contributor to the onset of apathy in PD, which was verified by a previous study using a single-photon emission CT (Santangelo et al., 2015a). These authors reported that after adjusting for age, disease duration, the site of onset of motor symptoms, and the severity of motor symptom, the striatal levels of dopamine transporter were lower in untreated patients with PD with apathy than in those without apathy. Pharmacological studies (Czernecki et al., 2002; Thobois et al., 2013) that reported an improvement in apathy following dopaminergic treatment also partly support the role of dopaminergic deficit in the development of apathy in PD.

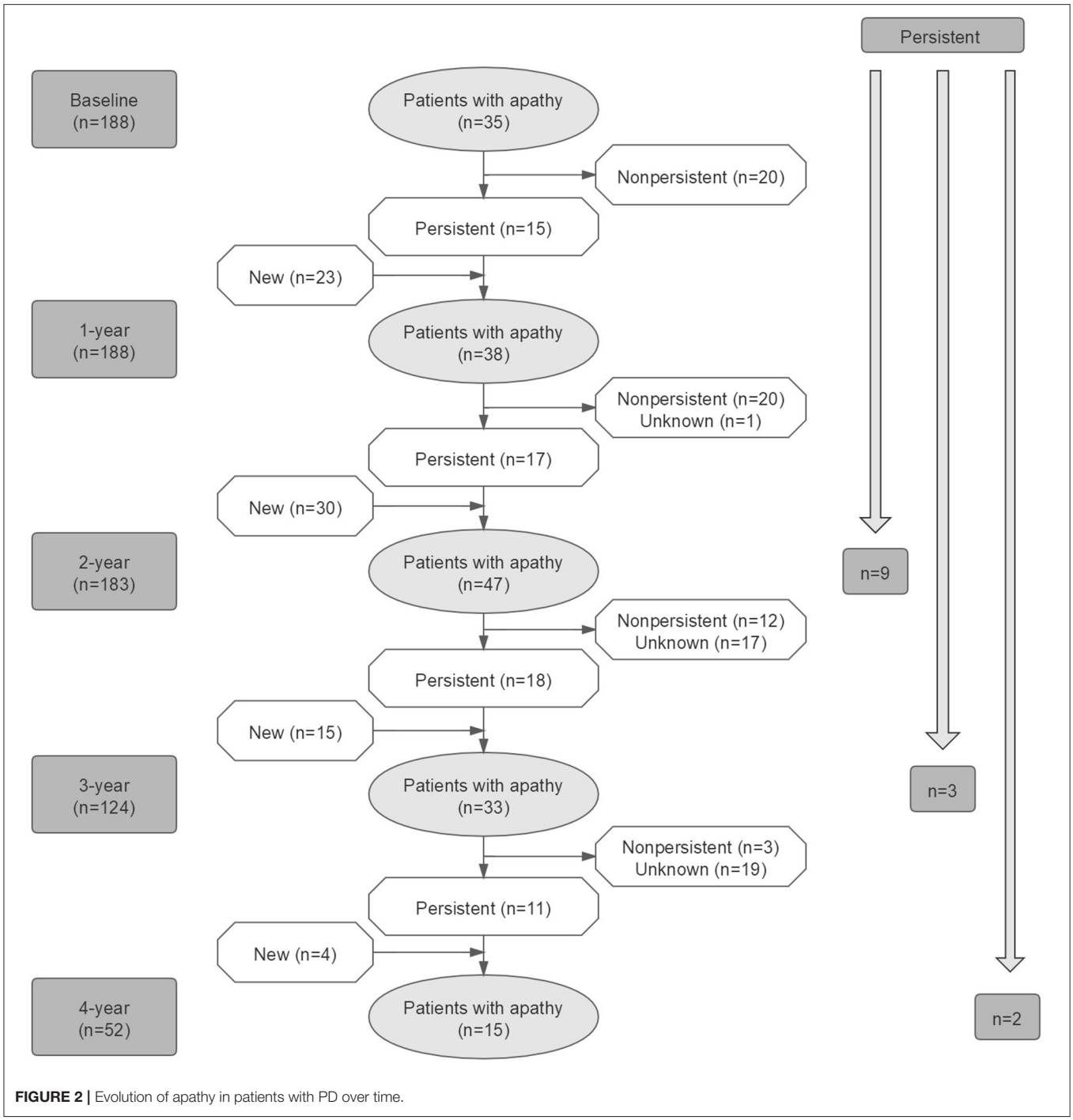

FIGURE 2 | Evolution of apathy in patients with PD over time.

However, we found that the LEDD changes were not different between patients with and without persistent apathy, and no association was observed between the LEDD and the LARS score, suggesting that the non-dopaminergic system may also play a role in apathy.

A previous study reported that patients with PD presenting with apathy show the impairment of global cognitive function and diminished the ability to perform cognitive tasks (Pagonabarraga et al., 2015). In the current study, we focused on the effect of each cognitive subdomain on apathy. Apathy was significantly and independently associated with a decline in attention and executive functions, which was inconsistent with the results of a previous cross-sectional study (Pedersen et al., 2010) in which the authors did not observe any association between apathy and cognitive dysfunction, including the following cognitive domains: memory, attention/executive

TABLE 2 | Factors associated with higher LARS scores in patients with PD.

	Unadjusted model			Adjusted model		
	B	**95%CI**	***P*-value**	**B**	**95%CI**	***P*-value**
Age	1.022	0.927–1.126	0.662	0.946	0.875–1.022	0.160
Disease duration	1.404	0.961–2.053	0.080			
Male sex	7.104	1.172–43.051	0.033*	8.131	1.673–39.521	0.009*
Education	0.767	0.606–0.970	0.027*	0.944	0.784–1.137	0.542
LEDD	1.003	1.000–1.006	0.049*	0.997	0.994–1.000	0.055
Use of levodopa	2.923	0.730–11.711	0.130			
Use of dopamine agonist	1.936	0.436–8.584	0.385			
Use of antidepressant	29.859	0.670–1329.944	0.080	6.360	0.196–206.753	0.298
FAB	0.462	0.286–0.746	0.002*	0.567	0.366–0.879	0.011*
MOCA	0.626	0.482–0.812	<0.001*			
Visuospatial/executive ability	0.365	0.199–0.671	0.001*			
Naming	0.428	0.118–1.558	0.198			
Attention	0.100	0.024–0.420	0.002*	0.217	0.056–0.833	0.026*
Language	0.348	0.125–0.969	0.043*	0.726	0.305–1.730	0.470
Abstraction	0.916	0.310–2.701	0.873			
Memory	0.785	0.468–1.316	0.359			
Orientation	0.114	0.022–0.575	0.009*	0.582	0.148–2.287	0.483
HDRS	1.409	1.248–1.591	<0.001*	1.362	1.176–1.577	<0.001*
HARS	1.329	1.153–1.532	<0.001*	0.959	0.784–1.174	0.688
UPDRS III	1.232	1.138–1.335	<0.001*	1.147	1.064–1.236	<0.001*
H&Y	57.108	8.785–371.264	<0.001*			
Follow-up time in years	1.734	1.040–2.892	0.035*	1.785	1.005–3.168	0.048*

PD, Parkinson's disease; LARS, Lille Apathy Rating Scale; LEDD, levodopa equivalent daily dose; FAB, frontal assessment battery; MOCA, Montreal Cognitive Assessment; HDRS, Hamilton Depression Rating Scale; HARS, Hamilton Anxiety Rating Scale; UPDRS, Unified Parkinson's Disease Rating Scale.
**Significant difference.*

TABLE 3 | Predicted factors for the development of apathy in patients with PD ($n = 153$).

	Unadjusted model			Adjusted model		
	HR	**95%CI**	***P*-value**	**HR**	**95%CI**	***P*-value**
Age	0.987	0.960–1.014	0.345			
Female sex	0.718	0.433–1.190	0.198	0.729	0.438–1.213	0.224
Education	0.985	0.928–1.045	0.609			
FAB	0.908	0.820–1.007	0.066	1.004	0.904–1.116	0.937
MOCA	0.928	0.879–0.979	0.006*			
Visuospatial/executive ability	0.855	0.717–1.020	0.082			
Naming	0.746	0.506–1.099	0.139			
Attention	0731	0.546–0.980	0.036*	0.795	0.563–1.124	0.194
Language	0.718	0.539–0.956	0.023*	0.794	0.587–1.076	0.137
Abstraction	0.942	0.687–1.290	0.708			
Memory	0.935	0.789–1.108	0.440			
Orientation	0.879	0.644–1.201	0.418			
HDRS	1.046	1.006–1.087	0.024*	1.043	1.005–1.081	0.026*
HARS	1.030	0.978–1.085	0.264			
UPDRS III	1.026	1.003–1.049	0.026*	1.016	0.994–1.038	0.157
H&Y	1.660	1.173–2.350	0.004*			

PD, Parkinson's disease; FAB, frontal assessment battery; MOCA, Montreal Cognitive Assessment; HDRS, Hamilton Depression Rating Scale; HARS, Hamilton Anxiety Rating Scale; UPDRS, Unified Parkinson's Disease Rating Scale.
**Significant difference.*

functions, psychomotor speed, and visuospatial skills. The association between apathy, attention, and executive dysfunction indicates possible underlying pathophysiological mechanisms that are common to these symptoms. A previous study that investigated apathy in patients with neurodegenerative conditions reported that patients with apathy showed impaired attention (Guimaraes et al., 2014). A prospective longitudinal study that included drug-naïve patients with PD (Santangelo et al., 2015b) also reported that baseline executive dysfunction was more severe in patients with PD presenting with apathy than in those without apathy, which suggests that poor performance on the Stroop test (which evaluates prefrontal cortex function) predicts the development of apathy after 2-years follow-up. Another study showed that repetitive transcranial magnetic stimulation improved the scores of patients' on the Stroop test, which suggests that executive and attention functions are associated with the frontal lobe activity (Boggio et al., 2005). A neuroimaging study (Benoit and Robert, 2011) showed that apathy in PD was associated with a reduction in gray matter volume or functional deficits in many regions, including the anterior and posterior cingulate cortices and dorsolateral or inferior frontal gyri. These findings suggest a strong association between apathy and prefrontal cortex dysfunctions, as identified in patients with other neurodegenerative diseases, such as Alzheimer's disease (Grossi et al., 2013). Moreover, the depletion of a cholinergic neuron has been implicated as an important contributor to cognitive dysfunction. This finding is supported by a double-blind, placebo-controlled study (Devos et al., 2014), which reported that rivastigmine could significantly improve the LARS score in patients with apathy but without dementia and depression. However, owing to the small number of patients who received the acetylcholinesterase inhibitor (AChE-I) treatment in our cohort, we did not investigate the effect of AChE-I on apathy in the current study.

Notably, men with PD were more likely to show apathy. A prospective longitudinal study (Wee et al., 2016) that included patients with PD observed that the progression of apathy was more rapid in men than in women. Our study indicates that apathy in PD could be considered a predictor of poor prognosis in men. However, the association between apathy and male sex should be considered with caution because apathy is more commonly observed in women than in men in the general population (Clarke et al., 2010). Further pathophysiological studies are warranted to verify the issue.

Another important finding in our study is that apathy was associated with depression scores, which is consistent with the results of two previous cross-sectional studies (Pedersen et al., 2010; Den Brok et al., 2015). It is reasonable to conclude that dysfunction of the dopaminergic mesocorticolimbic system, which is known to play a central role in the control of mood and motivation, is a common feature in both apathy and endogenous depression (Marin, 1991). A previous study in which PET (Maillet et al., 2016) was performed in patients with drug-naïve PD has proved the prominent role of serotonergic degeneration in the expression of apathy and depression. Conventionally, apathy is considered an aspect of depression; therefore, the association between depression scores and a high risk of apathy is not unexpected. Although apathy and depression are both commonly associated with PD (Den Brok et al., 2015), and both usually show overlapping symptoms (such as lack of interest), research has confirmed that apathy and depression can exist as distinct entities in patients with PD (Zahodne et al., 2012; Skidmore et al., 2013). However, since we found that apathy was not persistent in the current study, the predicted effect of depression on apathy is likely to be unstable and needs to be confirmed by further studies.

The limitations of this study are as follows: (A) The study did not include a group of healthy individuals (controls) against whom we could compare the progression of apathy in patients with PD. (B) This was a single-center study (all patients were recruited only from a single tertiary referral center in southwest China); therefore, our results should be confirmed by future multi-center studies. (C) Nearly 50% of the patients received baseline drug treatment; therefore, we could not verify the progression of "pure" apathy in patients with PD. (D) The relatively short period during which disease progression occurred in some patients is insufficient to determine the long-term evolution of apathy in PD. (E) We did not use specific instruments (or tools) to assess cognition. (F) Some patients did not reach the time to 4-years follow-up visit ($n = 49$), which contributed to the relatively small number of patients we included at that time ($n = 52$).

In conclusion, our study showed that the prevalence of apathy is higher in patients with progressive PD and that apathy is associated with male sex and disease-specific symptoms, including motor and non-motor symptoms. We observed that depression in early-stage PD is the main predictor of apathy in patients with PD. Our study highlights that apathy is an early, common, and non-persistent non-motor symptom in patients with early PD and that this finding may have implications for clinical management.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author (Huifang Shang, E-mail address: hfshang2002@126.com) upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Sichuan University West China Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RO, JL, and HS contributed to planning the study. RO contributed to data analysis and drafting of the manuscript. RO, JL, KL, ZJ, QW, YH, LZ, BC, BZ, and WS contributed to the recruitment of patients and processing

of follow-up visits. All authors contributed to revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.620762/full#supplementary-material

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The Relationship Between Retinal Nerve Fiber Layer Thickness and Clinical Symptoms of Alzheimer's Disease

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Background/Aim: Retinal nerve fiber layer (RNFL) thickness (RT), which can reflect the status of the retinal optic nerve cells, may be affected in patients with Alzheimer's disease (AD). There are few studies on the correlation of RT of patients with AD (AD-RT) with clinical symptoms of various cognitive domains, neuropsychiatric symptoms, and activities of daily living (ADL). This study is to investigate the relationships between RT and the abovementioned clinical symptoms of AD.

Methods: A total of 96 patients with AD were included in this study. RT was measured in these patients using optical coherence tomography (OCT). Demographic variables, RT, and clinical symptoms were compared between the normal and the abnormal AD-RT groups. Clinical symptoms, including cognitive symptoms, neuropsychiatric symptoms, and ADL, were evaluated using a series of rating scales.

Results: The relationships between RT and cognitive symptoms scores were analyzed in patients with AD. Reduced RT was found in 54.4% of patients with AD. The average RT, RT of the superior 1/2 quadrant, and RT of the inferior 1/2 quadrant of both eyes were all significantly decreased in the abnormal AD-RT group ($p < 0.001$). Overall cognitive function and performance in multiple cognitive domains, including memory, language, attention, and executive function, were also significantly impaired in the abnormal AD-RT group ($p < 0.05$). For lower RT value, the global cognitive function and the performance in multiple cognitive domains were worse. ADL was significantly compromised in patients with AD having lower RT values ($p < 0.05$).

Conclusions: Lower RT value appear to be correlated with cognitive impairment, and RT may be an indicator of cognitive decline in patients with AD. Further studies are required to confirm our findings.

Keywords: Alzheimer disease, retinal nerve fiber layer thickness, optical coherence tomography, clinical features, cognitive level

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INTRODUCTION

The retina is a peripheral extension of the central nervous system. It has a similar tissue source and an anatomical structure to the central nervous system, including neurons, ganglion cells, and a blood barrier (Maccormick et al., 2015; Trost et al., 2016; Diaz-Coranguez et al., 2017). The typical pathological hallmarks of Alzheimer's disease (AD), including the accumulation of amyloid plaques and neurofibrillary tangles, can affect the relevant regions of the visual cortex in the early stage of the disease (Sperling et al., 2011). Retinal nerve fiber layer (RNFL) thickness (RT), which can reflect the status of the retinal optic nerve cells, may also be affected in patients with AD. Moreover, degeneration in the RNFL has been shown to parallel disease severity in patients with AD (Liu et al., 2015; Garcia-Martin et al., 2016). Research has shown that RT is lower in patients with AD than in normal elderly individuals (Gao et al., 2015). Interestingly, RNFL thinning has been observed in the early stage of dementia and the mild cognitive impairment (MCI) stage of patients with AD, suggesting the potential value of RNFL thinning in the early identification of AD (Holroyd and Shepherd, 2001). Some studies have not found a significant association of retinal thinning with lower Mini–Mental State Examination (MMSE) score (Cipollini et al., 2020), while other studies showed a significant correlation between the MMSE score and the RNFL value (Oktem et al., 2015). Studies reported the correlation between RT and the decline of the overall cognitive function, indicating that the thinner the RT, the worse the overall cognitive function (Tzekov and Mullan, 2014; Cheung et al., 2015). Research found that the reduction in the magnitude of macular RNFL volume significantly correlated with the performance of participants' abilities on a task to efficiently integrate visual and auditory speech information (Santos et al., 2018). A study on a few samples showed that RT correlated with the neuropsychological performance in multiple cognitive domains (e.g., working memory, psychomotor speed, and executive function; Mammadova et al., 2020). However, there are a few studies that have examined the correlation between RT in patients with AD (AD-RT) and various cognitive domains, including memory, attention, language, visuospatial ability, and executive function, as well as neuropsychiatric symptoms, such as anxiety, depression, and agitation.

In this study, RT was measured using optical coherence tomography (OCT), and the clinical characteristics of AD-RT were analyzed. Demographic variables were collected and clinical symptoms, including cognitive function, neuropsychiatric symptoms, and activities of daily living (ADL), were evaluated using a series of rating scales. The relationships between RT and demographic variables and clinical symptoms of patients with AD were analyzed. This investigation aimed to provide a clinical basis for understanding the changes of RT in patients with AD and its correlation with the clinical symptoms of AD.

MATERIALS AND METHODS

Ethics Statement

This study met the guidelines on ethical principles for medical research involving human subjects of the Declaration of Helsinki, and the study protocol was approved by the Ethical Review Board of Beijing Tiantan Hospital. Written informed consent was obtained from patients and their family members. All methods were performed following relevant guidelines and regulations.

Subjects

Inclusion Criteria

This study included patients with mild cognitive impairment (MCI) due to AD (Albert et al., 2011) and patients with AD dementia (Mckhann et al., 2011) according to the National Institute of Aging and Alzheimer's Association (NIA-AA) criteria.

Exclusion Criteria

The exclusion criteria of this study were as followed: (1) the presence of one or more of the following ophthalmic diseases: glaucoma, cataract, optic neuropathy, retinal vascular disease, retinal detachment, and macular degeneration; (2) the presence of high myopia $>600^{\circ}$, pupil <2 mm; (3) a history of ocular trauma; (4) previous ophthalmological surgeries performed within the previous 6 months; (5) the presence of systemic diseases, including hypothyroidism, severe chronic diseases, and other medical diseases that might affect vision; and (6) a history of alcoholism or carbon monoxide poisoning.

Collection of Demographic Information

Demographic variables, including gender, age, age of onset of AD, disease duration, and education level, were recorded for all participants with AD.

Evaluation of RT by OCT

The patient was seated in a quiet state and scanned using an RTVue 100 OCT machine (Optovue, Inc., Fremont, CA, USA), with the optic nerve head (ONH) scanning mode. Optic nerve head-centered circular scanning was performed at a depth of 5 μm and a diameter of 3.45 mm. The scanning proceeded through each quadrant in turn. The right eye was scanned clockwise, while the left eye was scanned counter-clockwise. RT value was generated after automatic mapping and evaluation using a computer program. Ganglion cell complex thickness (GCCT) of each eye was also evaluated. Repeated scans were performed three times, and the average value was taken as AD-RT. Both eyes were scanned by the same ophthalmologist who did not know the patient's diagnosis, and the images and results were saved.

The measured quadrants included the superior nasal (SN), nasal upper (NU), nasal lower (NL), inferior nasal (IN), inferior temporal (IT), temporal lower (TL), temporal upper (TU), and superior temporal (ST) quadrants, in both oculus dexter (OD), and oculus sinister (OS). The superior 1/2 quadrants included the SN, NU, TU, and ST quadrants, and the inferior 1/2 quadrants included the NL, IN, IT, and TL quadrants. After scanning

the eyes of the patients with AD using OCT, the computer image analysis system compared the RT values obtained with the normal values in the database for the same age, sex, eye, and part, and color-coded it green, yellow, and red to represent the confidence intervals of 95–5%, 5–1%, and 1–0% of the normal population, respectively (**Supplemantery 1**).

Assessment of Cognitive Function

Global Cognitive Function

Mini-mental State Examination (MMSE) scale was used to evaluate the global cognitive function of patients with AD. The lower the score of the MMSE scale, the more severe the cognitive impairment.

Cognitive impairment was established in patients with illiteracy, primary education, and junior and higher education when the MMSE score was below 17, 20, and 24 points, respectively. It is one of the standards for the Chinese version of MMSE, which cutoff value was formulated by Zhang (1995).

Individual cognitive domains were assessed using the following rating scales:

Memory

Visual delayed memory was evaluated by the Rey-Osterreith Complex Figure Test (CFT)-delayed memory (Guo et al., 2009). Low score of this scale indicated poor verbal and visual memories.

Visuospatial Ability

Visuospatial ability was evaluated using the CFT-imitation (Guo et al., 2009). A low score in this test suggested worse visuospatial ability.

Language

Language function was evaluated using the Animal Fluency Test (AFT; Lin et al., 2014). A low score implied compromised language function.

Attention

Attention was evaluated using the Trail Making Test A (TMT-A;Wei et al., 2018). The longer it took to complete the test, the worse an individual's attention.

Executive Function

Executive function was rated using the Stroop Color-Word Test (SCWT;Bondi et al., 2002). A low score of this test indicated an impaired executive function.

Assessment of Neuropsychiatric Symptoms

Overall neuropsychiatric symptoms were assessed using the Neuropsychiatric Inventory (NPI). A high score implied severe overall neuropsychiatric symptoms.

Individual neuropsychiatric symptoms were then assessed using the following rating scales:

Depression

Depression was evaluated using the Hamilton Depression Scale (HAMD)-24 items. Higher score indicated more severe depression, and a score of $\geq$8 confirms the presence of depression.

Anxiety

Anxiety was evaluated using the Hamilton Anxiety Scale (HAMA)-14 items. An elevated score of this test suggested more severe anxiety, and a score of $\geq$8 confirmed the presence of anxiety.

Agitation

Agitation was rated using the Cohen-Mansfield Agitation Inventory (CMAI). The higher the CMAI score, the more severe the agitation.

Apathy

Apathy was rated using the Modified Apathy Estimate Scale (MAES). The higher the score, the more severe the apathy. A score of >14 indicated clinically meaningful apathy.

Assessment of the Activities of Daily Living

Activities of daily living were assessed using the ADL scale, which includes the basic (ADL) (BADL) and the instrumental ADL (IADL) scales. The higher the score, the worse the ADL performance (Zhang, 1995).

Data Analyses

Statistical analyses were performed using the SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA). A value of $p < 0.05$ was considered statistically significant.

Continuous variables, if normally distributed, are presented as means ± SDs and were compared using the two-sample *t*-test. Continuous variables, if they were not normally distributed, are presented as medians (quartiles) and were compared using a non-parametric test. The bivariate correlation method was used to analyze the correlation of the measurement data.

Demographic variables, RT, and clinical symptoms were compared between the normal and the abnormal AD-RT groups. Clinical symptoms, including cognitive symptoms, neuropsychiatric symptoms, and ADL determined by the corresponding rating scales, were compared between the normal and the abnormal AD-RT groups.

Spearman correlation analyses were performed between the RT and the scores of cognitive symptoms, neuropsychiatric symptoms, and ADL in patients with AD.

RESULTS

The Frequency of Abnormal RT in Patients With AD

Of the 96 patients with AD included in this study, 52 (54.17%) had an abnormal RT and 44 (45.83%) had a normal RT, as revealed by OCT.

Comparison of RT Between the Normal and the Abnormal AD-RT Groups

The RTs of the right and left eyes in the normal and the abnormal AD-RT groups were compared. Compared to the normal AD-RT group, the average RT, the RT of the superior 1/2 quadrants,

TABLE 1 | Comparison of retinal nerve fiber layer (RNFL) thickness (RT) and ganglion cell complex thickness (GCCT) between the normal and the abnormal RT of patients with AD (AD-RT) groups.

	Normal AD-RT group (44 cases)	Abnormal AD-RT group (52 cases)	*p*
Right eye			
Average RT [μm, median (quartile)]	109.82 (101.50, 115.53)	95.25 (91.06, 103.94)	<0.001**
RT of the superior 1/2 quadrant (μm, mean ± SD)	109.86 ± 10.76	97.40 ± 12.97	<0.001**
RT of the inferior 1/2 quadrant (μm, mean ± SD)	107.85 ± 11.27	95.76 ± 11.73	<0.001**
Left eye			
Average RT (μm, mean ± SD)	109.26 ± 9.37	96.70 ± 9.39	<0.001**
RT of the superior 1/2 quadrant (μm, mean ± SD)	112.09 ± 10.16	99.09 ± 12.06	<0.001**
RT of the inferior 1/2 quadrant [μm, median (quartile)]	107.49 (99.29, 112.47)	94.05 (87.79, 99.11)	<0.001**
Right eye			
Average GCCT (μm, mean ± SD)	91.07 ± 15.95	87.3 ± 8.13	0.144
GCCT of the superior 1/2 quadrant (μm, mean ± SD)	91.05 ± 16.06	87.82 ± 9.29	0.228
GCCT of the inferior 1/2 quadrant (μm, mean ± SD)	90.86 ± 16.02	85.22 ± 13.66	0.069
Left eye			
Average GCCT (μm, mean ± SD)	92.77 ± 8.56	84.92 ± 20.56	0.022*
GCCT of the superior 1/2 quadrant (μm, mean ± SD)	92.42 ± 8.15	84.92 ± 20.31	0.026*
GCCT of the inferior 1/2 quadrant (μm, mean ± SD)	93.07 ± 9.45	83.74 ± 21.38	0.01*

$^{**}p < 0.01$, $^{*}p < 0.05$.

and the RT of the inferior 1/2 quadrants of the right and left eyes were all significantly decreased in the abnormal AD-RT group ($p < 0.001$; **Table 1**). The average GCCT, the GCCT of superior 1/2 quadrant, and the GCCT of the inferior 1/2 quadrant of left eye were all significantly decreased in the abnormal AD-RT group ($p < 0.05$; **Table 1**).

Relationship Between RT and Demographic Variables

Demographic variables, including sex, age, age of onset, disease duration, and education level were compared between the normal and the abnormal AD-RT groups. No significant differences in the demographic variables were observed between the two groups (**Table 2**).

Relationship Between RT and Cognitive Function

First, the relationship between RT and overall cognitive function was analyzed. The MMSE score in the abnormal AD-RT group was significantly lower than that in the normal AD-RT group ($p < 0.01$; **Table 3**).

The correlation between RT and MMSE score was then analyzed. The results indicated that the average RT and the RT of the superior 1/2 quadrant of the right eye were significantly and positively correlated with the MMSE score in patients with AD (**Table 4**), indicating that in patients with AD, the lower the RT value, particularly the RT of the superior 1/2 quadrants of the right eye, the worse the overall cognitive function (**Table 4**).

Secondly, the relationship between the RT and individual cognitive domains was analyzed.

Memory

The CFT-delayed memory score in the abnormal AD-RT group was significantly lower than that in the normal AD-RT group (**Table 3**), indicating that the visual delay recall function of the abnormal AD-RT group was significantly compromised when compared with the normal AD-RT group ($p < 0.01$; **Table 3**). The average RT and the RT of the superior 1/2 quadrant of the right eye were significantly and positively correlated with the CFT-delayed memory score in patients with AD (**Table 5**), illustrating that the lower the RT value, particularly the RT of the superior 1/2 quadrant of the right eye, the more severe the visual delayed recall impairment in patients with AD.

Language

The AFT score in the abnormal AD-RT group was significantly lower than that in the normal AD-RT group (**Table 3**), demonstrating that the language function of the abnormal AD-RT group was significantly damaged. The AFT scale score was significantly and positively correlated with the average RT, the RT of the superior 1/2 quadrants, and the RT of the inferior

TABLE 2 | Comparisons of demographic variables between the normal and the abnormal AD-RT groups.

	Normal AD-RT group (44 cases)	Abnormal AD-RT group (52 cases)	*p*
Sex			
Male [cases (%)]	16 (36.36%)	23 (44.23%)	0.434
Female [cases (%)]	28 (63.64%)	29 (55.77%)	
Age (years, mean ± SD)	65.98 ± 9.307	69.6 ± 10.01	0.078
Age of onset (years, mean ± SD)	63.43 ± 9.195	65.07 ± 12.005	0.479
Disease duration [years, median (quartile)]	3.00 (2.00, 4.00)	3.00 (2.00, 5.00)	0.144
Education level [cases (%)]			
Primary school and below	11 (25.00%)	13 (25.00%)	0.213
Middle and high school	18 (40.91%)	29 (55.77%)	
Bachelor's degree and above	15(34.09%)	10 (19.23%)	

TABLE 3 | Comparison of cognitive function between the normal and the abnormal AD-RT groups.

	Normal AD-RT group (44 cases)	Abnormal AD-RT group (52 cases)	*p*
Global cognitive function			
MMSE [scores, median (quartile)]	25.00 (18.00, 29.00)	21 (14.00, 26.00)	0.036*
Cognitive domain			
Memory			
CFT-delayed memory [scores, median (quartile)]	3.00 (0.00, 10.00)	0.00 (0.00, 4.50)	0.032*
Language			
AFT [scores, median (quartile)]	14.00 (9.00, 20.00)	9.5 (6.00, 14.75)	0.003*
Attention			
TMT-A-time [seconds, median (quartile)]	82.00 (60.00, 125.50)	129.00 (66.00, 220.50)	0.033*
Visuospatial ability			
CFT-imitation [scores, median (quartile)]	22.00 (6.25, 34.00)	14.00 (2.00, 28.00)	0.122
Executive function			
SCWT-time [seconds, median (quartile)]	77.00 (64.50, 105.00)	110.00 (74.00, 161.00)	0.006*

*$p < 0.05$.

MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; CFT, Complex Figure Test; AFT, Animal Fluency Test; TMT, Trial Making Test; SCWT, Stroop Color Word Test.

1/2 quadrants of the right eye in patients with AD (**Table 5**), demonstrating that in patients with AD, the lower the RT value of the right eye, the worse the language function.

Attention

The time taken to complete the TMT-A in the abnormal AD-RT group was significantly longer than that in the normal AD-RT group ($p < 0.01$; **Table 3**), illustrating that attention in the abnormal AD-RT group was markedly impaired relative to that in the normal AD-RT group.

Executive Function

The time taken to complete the SCW scale in the abnormal AD-RT group was significantly longer than that in the normal AD-RT group ($p < 0.01$; **Table 3**), illustrating that the executive function in the abnormal AD-RT group was significantly damaged relative to that in the normal AD-RT group. The time taken to complete the SCW scale was significantly and negatively correlated with the average RT, the RT of the superior 1/2 quadrant, and the RT of the inferior 1/2 quadrant of the left eye (**Table 5**), implying that, in patients with AD, the lower the RT of the left eye, the worse the executive function.

Visuospatial Ability

There were no significant differences in the Rey Complex Figure Test (RCFT) and the Clock Drawing Test (CDT) scores between the normal and the abnormal AD-RT groups (**Table 3**), demonstrating that RT was unrelated to the visuospatial ability in patients with AD in our study.

Relationship Between RT and Neuropsychiatric AD Symptoms

First, the relationship between the RT and the total neuropsychiatric symptoms was analyzed. There was no significant difference in the NPI score between the normal and the abnormal AD-RT groups (**Table 6**), demonstrating that the RT was not related to the total neuropsychiatric of AD symptoms.

Second, the relationship between the RT and the individual neuropsychiatric symptoms were analyzed. There were no significant differences in the HAMD, the HAMA, the CMAI, and the MAES scale scores (**Table 6**), demonstrating that the RT was not related to depression, anxiety, agitation, or apathy in patients with AD.

TABLE 4 | Correlation analysis between RT and global cognitive function of patients with AD.

	Score of MMSE scale	
	R	*p*
Right eye		
Average RT	0.232	0.023*
RT of superior 1/2 quadrant	0.261	0.001*
RT of inferior 1/2 quadrant	0.144	0.163
Left eye		
Average RT	0.147	0.153
RT of superior 1/2 quadrant	0.185	0.070
RT of inferior 1/2 quadrant	0.045	0.660

*$p < 0.05$.

Relationship Between RT and ADL

We compared the ADL scale scores between the normal and the abnormal AD-RT groups, and the results demonstrated that the ADL score in the abnormal AD-RT group was significantly higher than that in the normal AD-RT group [26.00 (20.00, 37.00) vs. 20.00 (20.00, 25.00), $p < 0.01$], illustrating that the ADL score in the abnormal AD-RT group was significantly lower than that in the normal AD-RT group. The ADL score was significantly and negatively correlated with the average RT of the right eye in patients with AD (**Table 7**), demonstrating that the lower the average RT values of the right eye, the worse the ADL score of patients with AD.

TABLE 5 | Correlation analysis between RT and individual cognitive domain in patients with AD.

	Score of CFT-delayed memory		Score of AFT scale		Time used for TMT-A		Time used for SCWT	
	r	*p*	r	*p*	R	*p*	r	*p*
Right eye								
Average RT	0.385	0.001**	0.294	0.004**	−0.053	0.634	−0.152	0.200
RT of superior 1/2 quadrant	0.368	0.002**	0.298	0.003**	−0.132	0.236	−0.203	0.085
RT of inferior 1/2 quadrant	0.310	0.009**	0.230	0.024*	0.045	0.691	−0.07	0.555
Left eye								
Average RT	0.308	0.010*	0.271	0.008**	−0.083	0.457	−0.222	0.059
RT of superior 1/2 quadrant	0.337	0.005**	0.237	0.020*	−0.158	0.156	−0.182	0.123
RT of inferior 1/2 quadrant	0.224	0.065	0.209	0.041*	−0.001	0.991	−0.238	0.042*

*$p < 0.05$, **$p < 0.01$.

TABLE 6 | Comparisons of neuropsychiatric symptoms between the normal and the abnormal AD-RT groups.

	Normal AD-RT group (44 cases)	Abnormal AD-RT group (52 cases)	*p*
NPI [scores, median (quartile)]	1.50 (0.00, 8.25)	1.00 (0.00, 4.00)	0.996
HAMD [scores, median (quartile)]	4.50 (2.00, 9.00)	7.77 ± 7.29	0.172
HAMA [scores, median (quartile)]	4.00 (1.25, 6.00)	5.00 (2.00, 10.00)	0.110
CMAI [scores, median (quartile)]	29.00 (29.00, 29.00)	29.00(29.00, 29.00)	0.498
MAES (scores, mean ± SD)	12.66 ± 10.94	16.89 ± 12.52	0.094

NPI, Neuropsychiatric Inventory; HAMD, Hamilton Depression Scale; HAMA, Hamilton Anxiety Scale; CMAI, Cohen-Mansfield Agitation Inventory; MAES, Modified Apathy Evaluation Scale.

TABLE 7 | Correlation analysis of RT and ADL in patients with AD.

	Score of ADL scale	
	r	*p*
Right eye		
Average RT	−0.383	<0.001**
RT of superior 1/2 quadrant	−0.410	<0.001**
RT of inferior 1/2 quadrant	−0.275	0.011*
Left eye		
Average RT	−0.273	0.012*
RT of superior 1/2 quadrant	−0.328	0.002*
RT of inferior 1/2 quadrant	−0.128	0.246

*$p < 0.05$, **$p < 0.01$.

DISCUSSION

Abnormal RT has not attracted an extensive attention from clinicians or patients with AD. Therefore, the frequency of abnormal AD-RT has rarely been reported. In this study, OCT was used to measure the RT of patients with AD. According to the diagnostic criteria adjusted for sex and age, the results demonstrated that the frequency of the abnormal AD-RT was 54.16%. The RT can be influenced by many factors, for example, age and vascular risk factors (Gattoussi et al., 2019). It appears to be correlated with cognitive impairment, which was shown in our study. In the present study,there were 44 cases (45.83%) of all AD patients with normal RT, which was speculated that normal AD-RT group had higher global cognitive function.

At present, there is no unified conclusion in the study of RT among patients with AD. The RT of the superior 1/4 quadrant (ST+SN regions) and the inferior 1/4 quadrant (IT+IN regions) have been shown to be reduced in patients with AD, while no significant changes were observed in other retinal areas (Lu et al., 2010). It has also been shown that the average, superior, and inferior RT were significantly decreased in patients with AD (Ngoo et al., 2019). The global and temporal superior quadrants' peripapillary RNFL and the superior pericentral and peripheral sectors of the overall RT in patients with AD have been shown to be significantly thinner (Cunha et al., 2017). In this study, the RT of the superior 1/2 quadrant and of the inferior 1/2 quadrant and the average RT of each eyes were all significantly decreased. Studies have found that the decrease in RT in the superior 1/2 quadrant in patients with AD might be caused by the following reasons: the axons of the RNFL in the superior 1/2 quadrant reach the cuneus gyrus of the primary optic cortex through the optic radiation, and the axons of the RNFL in the inferior 1/2 quadrant reach the lingual gyrus through the optic radiation. It has been reported that the densities of the neuroinflammatory response in amyloid plaques and neurofibrillary tangles in the cuneus gyrus are significantly higher than those in the lingual gyrus; therefore, the RT of the superior 1/2 quadrants was more prone to be thinner (Armstrong, 1996). Additionally, we observed that the RT of the inferior 1/2 quadrant was significantly decreased; thus, the average RT was decreased because both the superior 1/2 and inferior 1/2 quadrants were reduced. It has been suggested that visual abnormalities in patients with AD might be associated with neurodegeneration in the visual cortex, and the neuroinflammatory response in amyloid plaques as well as the neurofibrillary tangles might occur in the visual cortex in the early stage of AD—even earlier than in the hippocampus (Mckee et al., 2006). Amyloid precursor protein (APP) is synthesized in retinal ganglion cells and rapidly transported into the optic nerve in small transport vesicles (Morin et al., 1993). Combined with the cognitive decline in the abnormal AD-RT group, we speculated that there might be more β amyloid (Aβ) deposition and more severe neurodegeneration. Thus, the RT of the superior 1/2 quadrant and of the inferior 1/2 quadrant and the average RT of both eyes might be dramatically reduced in the abnormal AD-RT group.

The relationship between RT and demographic variables was analyzed between the normal and the abnormal AD-RT groups. Similar to other studies, we failed to find a significant gender difference between the two groups. An early study reported that RT decreased with age (Balazsi et al., 1984), while another study reported a minimal effect of age on RT (Polito et al., 2002). A recent study showed that the RT of some sectors decreased with age (Jang et al., 2018), and age was not a constant confounder when using OCT (Hsu et al., 2012). We found no differences in age or age of onset between the two groups. Over time, Aβ and tau in the retina of patients with AD might gradually accumulate, and RT might progressively reduce. However, similar to other investigations, we did not observe different disease durations between the two groups. No previous study has focused on RT and the education level; here, we did not observe a difference in the education levels between the two groups. In conclusion, there were no differences in the abovementioned demographic variables between the two groups, indicating no relationship between RT and demographic variables.

The impaired cognitive domains in patients with AD mainly included memory, attention, language, visuospatial ability, and executive function.

The MMSE scale covers multiple cognitive domains and has high sensitivity for evaluating dementia and MCI. In this study, the overall cognitive function was assessed using the MMSE scale. The results demonstrated that the score of MMSE scale in the abnormal AD-RT group were significantly lower than those of the normal AD-RT group, and the average RT and the RT of the superior 1/2 quadrant of the right eye in patients with AD were significantly and positively correlated with the score of the MMSE scale; this illustrated that the overall cognitive function of the abnormal AD-RT group was dramatically impaired, and the lower the RT value, the more severe the cognitive decline in patients with AD.

Some studies have shown a significant correlation between the RT and the MMSE score (Iseri et al., 2006; Oktem et al., 2015), indicating that RT reduced with the aggravation of AD. While some investigations failed to find a significant association between retinal thinning and decreased MMSE score (Cipollini et al., 2019), this study showed that the MMSE score in the

abnormal AD-RT group was dramatically decreased compared to the normal AD-RT group, suggesting that the lower the RT value, the worse the cognitive function. A previous study showed that intravenous immunoglobulin might reduce Aβ depositions in retina and central nervous system (Kile et al., 2020). We speculated that the depositions of pathological proteins in the AD brain were increased, causing severe neuronal damage and subsequent overall cognitive impairment. Aβ and tau might accumulate in the retinas of patients with AD, progressively causing RT reduction.

Complex Figure Test-delayed memory is a commonly used rating scale for visual memory (Siri et al., 2001). Nonverbal spatial memory and imitation are often neglected in the clinical examination. Impairment of visual memory is one of the most important early manifestations of AD (Hayashi et al., 2018; Oltra-Cucarella et al., 2018). There has been no prior study evaluating the relationship between RT and CFT-delayed memory in patients with AD. In this study, the CFT-delayed memory score in the abnormal AD-RT group was significantly decreased, demonstrating that the visual delayed memory was dramatically damaged in the abnormal AD-RT group. In addition, the average RT and the RT of the superior 1/2 quadrant of the right eye were significantly and positively correlated with the CFT-delayed memory score, illustrating that the lower the RT value, the more severe the visual delayed memory impairment in patients with AD. Thus, the visual memory of patients with AD might be obviously affected by the reduction of RT, resulting in the visual delayed memory being dramatically impaired in the abnormal AD-RT group.

A verbal fluency test was used to investigate the relationship between RT and language impairment in patients with AD. Verbal fluency reflects instant verbal memory, spontaneous verbal motor ability, interference suppression ability, and thinking organization ability. In this study, the verbal fluency test required patients to list as many animal names as possible within 1 min. We found that the language function was dramatically damaged in the abnormal AD-RT group. Moreover, the average RT and the RT of the superior and the inferior 1/2 quadrants of the right eye were significantly and positively correlated with the score of verbal fluency test. AD is a neurodegenerative disease that extensively affects the cerebral cortex bilaterally; however, Aβ may form in different brain regions in the early stages, manifesting as different clinical types with different initial symptoms. Thus, language may be impaired when the lesions affect language-related regions of the brain. Patients with AD and amnestic MCI showing left-dominant hypometabolism tend to present severe impairment in verbal memory and be diagnosed with AD dementia (Murayama et al., 2016). Moreover, better cognitive performance has been shown to be significantly associated with increased RT in all tests, including a semantic fluency (animals and professions) test (Van Koolwijk et al., 2009). This study found evidence of an obvious language impairment in the abnormal AD-RT group. We speculated that the decrease in RT might compromise the transmission of retinal information to the occipital striate region, the primary visual cortex, which has extensive fibrous connections with the bilateral cerebral hemispheres that link the visual information to language processing. This might explain why the language was profoundly impaired in the abnormal AD-RT group.

The Trail Making Test A was used to explore the relationship between RT and attention in patients with AD. In this study, the time taken to complete the TMT-A in the abnormal AD-RT group was significantly prolonged, suggesting that attention was markedly impaired in this group. A previous study found that patients with AD had attention impairment in the early stage of the disease. Attention might be the second impaired cognitive domain after memory in patients with AD, manifesting earlier than the impairments in language and visuospatial ability (Perry et al., 2000). No previous study has examined the relationship between RT and attention in patients with AD. Optimal attention performance depends on the dorsal attention network (DAN) and the ventral attention network (VAN). DAN and VAN employ the dorsal frontoparietal areas (including the intraparietal sulcus, the frontal eye fields, etc.) and ventral frontoparietal areas (including the right-lateralized temporoparietal junction and ventral frontal cortex), respectively (Corbetta and Shulman, 2002; Fox et al., 2006). The brain regions associated with RT abnormalities include the temporal and occipital lobes. Accordingly, we speculated that there was an overlap between the regions related to attention impairment and abnormal RT, which might be the potential anatomical basis for attention impairment in the abnormal AD-RT group.

Complex Figure Test-imitation was used to investigate the relationship between RT and the visuospatial ability in patients with AD. Visuospatial impairment was a prominent early feature of clinically probable AD (Mandal et al., 2012). It has ventral and dorsal pathways. The ventral pathway starts from the occipital lobe and projects to the lower temporal cortex, which is mainly responsible for perceiving and identifying the objects seen by the eyes, as well as for the storage of the visuospatial memory in the medial temporal lobe and the hippocampus. The dorsal pathway also starts from the occipital lobe and projects to the parietal lobe, the prefrontal lobe, the cortical anterior motor region, and the medial temporal lobe, participating in the formation of visuospatial memory, visual navigation, and the subsequent processing of the visuospatial ability (Tales et al., 2005, 2011). Previous studies have demonstrated that patients with AD have visuospatial impairment (Yaari and Corey-Bloom, 2007; Mendez et al., 2018), but there has been no published research on the relationship between RT and the visuospatial ability. In this study, the two groups did not differ in visuospatial ability, which needs further investigation in large samples.

The Stroop Color-Word Test was used to evaluate the relationship between RT and the executive function in patients with AD. To the best of our knowledge, there has been no previous study on the relationship between AD-RT and executive impairment. In this study, the time taken to complete the SCWT in the abnormal AD-RT group was significantly prolonged, and the average RT and the RT of the superior and the inferior 1/2 quadrants of the left eye were significantly and negatively correlated with the time taken to complete the SCWT, illustrating that AD-RT was correlated with executive function. Executive function is an important cognitive domain that represents the control and processing capability of advanced

behavior. Executive dysfunction in AD included poor selective and divided attention, failed inhibition of interfering stimuli, and poor manipulation skills (Kirova et al., 2015). Executive function was mainly regulated by the frontal lobe (Alvarez and Emory, 2006). When eyes receive visual stimulation and transmit visual information from the optic nerve cells in the retina to the visual center, the whole visual neural network is activated between the primary visual cortex (occipital lobe) and the secondary cortex (the prefrontal lobe, the parietal lobe, the temporal lobe, spindle gyrus, and orbitofrontal gyrus). Lesions in the frontal lobe, a brain region that is vulnerable to AD, might destroy the visual neural network, which might be one of the reasons why the abnormal AD-RT group had executive impairment.

A body of neuropsychiatric symptoms in AD compromise the quality of life of a patient. Here, we analyzed the relationship between AD-RT and neuropsychiatric symptoms. The two groups did not differ in anxiety, depression, agitation, and apathy symptoms. Apathy was one of the commonest neuropsychiatric symptoms of AD, and significantly impaired the ADL of patients and increased the burdens of caregivers. (Sultzer, 2018). The structural integrity of the left anterior cingulate gyrus, the posterior cingulate gyrus, and the splenium, trunk and genu of the corpus callosum in patients with AD and apathy have been shown to be absent and significantly correlated with the severity of apathy (Hahn et al., 2013). We did not observe a correlation between RT and apathy, which might be due to different brain areas involved in apathy and reduced RT. A previous study reported that anxiety and depression in patients with AD were associated with a decreased metabolism in the parietal lobe. The subjective symptoms of depression were associated with high metabolism in the frontal lobe and low metabolism in the parietal lobe (Kotrla et al., 1995). Agitation was associated with atrophy of the frontal lobe, insula, amygdala, cingulate gyrus, and hippocampus (Trzepacz et al., 2013). The results of this study indicated that RT was not associated with the above neuropsychiatric symptoms. We will further explore the relationship between RT and other neuropsychiatric symptoms of AD, such as hallucination, illusion and delusion, in future work.

Finally, this study demonstrated that the ADL score in the abnormal AD-RT group was significantly increased, and there was a significant and negative correlation between the average RT of the right eye and the ADL score. The overall cognitive function, memory, attention, and executive function in the abnormal AD-RT group were severely damaged, which might result in significant impairment of ADL.

Despite the clinically interesting and potentially useful findings of this study, it has some limitations that should be noted. First, it was challenging to obtain the cerebrospinal fluid (CSF) from patients with AD who were elderly or who had either spinal deformities, bone hyperplasia, or other related conditions. The amyloid PET and/or fluorodeoxyglucose (FDG)-PET have not been covered by our medical insurance and are expensive for many families in China. Therefore, there was a lack of support for biomarkers (e.g., amyloid PET, CSF, and/or FDG-PET) in the criteria for the diagnosis of MCI due to AD or AD dementia adopted by this study. Second, in this study, RT assessed by OCT seemed not able to recognize patients with AD in almost 50% of the cases. In fact, more data on, for example, macular volume and vision are important regarding the visual recall and are pivotal for the results of cognitive tests and ADL. In the future, more data on the combined analyses of RT and GCCT need to be analyzed. Third, as half of our patients did not complete important MRI sequences, such as 3D-T1, diffusion tensor imaging, or functional MRI, the sample size was too limited to make robust conclusions from the neuroimaging assessments. Therefore, based on this study, we will undertake further research on the correlations between OCT values and neuroimaging results. Fourth, as a cross-sectional study, it is difficult to eliminate the effects of all the influencing factors. We are in the process of planning longitudinal studies that should be able to elucidate the progression of RT in patients with AD. Fifth, multiple comparisons may increase the probability of a type I error, especially in this study with a relatively small sample. However, the correction of multi-factor comparison may increase the probability of a type II error in this study. This is a preliminary exploration and large samples and the correction of multi-factor comparison are necessary in the future studies.

In conclusion, this study indicates that lower RT value may be correlated with cognitive impairment, and RT may serve as an indicator of cognitive decline in patients with AD. Further studies are required to confirm our findings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the ethics review board of Beijing Tiantan Hospital, Capital Medical University, Beijing. The patients/participants provided their written informed consents to participate in this study.

AUTHOR CONTRIBUTIONS

T-hL drafted the manuscript, carried out the analysis of data, accepted responsibility for the conduct of the research, final approval for the research, and performed the acquisition of data and the statistical analysis. ZJ drafted the manuscript, prepared the study design, carried out the analysis of data, accepted responsibility for the conduct of the research, provided final approval, and performed the acquisition of data and the statistical analysis. Y-zQ, W-jZ, and X-mW accepted responsibility for the conduct of the research and provided final approval. PG, D-yD, and D-nL carried out the acquisition of data, accepted responsibility for the conduct of the research, and provided final approval. H-yG drafted the manuscript, accepted responsibility for the conduct of the research, and provided final approval. L-xL carried out acquisition of data, accepted responsibility for

the conduct of the research, and provided final approval. WZ prepared the study design, carried out the analysis of data, accepted responsibility for the conduct of the research, provided final approval, and performed the acquisition of data, the statistical analysis, and study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2020.584244/full#supplementary-material

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BRIEF RESEARCH REPORT
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Reduced Facilitation of Parietal-Motor Functional Connections in Older Adults

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Age-related changes in cortico-cortical connectivity in the human motor network in older adults are associated with declines in hand dexterity. Posterior parietal cortex (PPC) is strongly interconnected with motor areas and plays a critical role in many aspects of motor planning. Functional connectivity measures derived from dual-site transcranial magnetic stimulation (dsTMS) studies have found facilitatory inputs from PPC to ipsilateral primary motor cortex (M1) in younger adults. In this study, we investigated whether facilitatory inputs from PPC to M1 are altered by age. We used dsTMS in a conditioning-test paradigm to characterize patterns of functional connectivity between the left PPC and ipsilateral M1 and a standard pegboard test to assess skilled hand motor function in 13 young and 13 older adults. We found a PPC-M1 facilitation in young adults but not older adults. Older adults also showed a decline in motor performance compared to young adults. We conclude that the reduced PPC-M1 facilitation in older adults may be an early marker of age-related decline in the neural control of movement.

Keywords: aging, posterior parietal cortex (PPC), primary motor cortex (M1), motor evoked potential, transcrancial magnetic stimulation

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INTRODUCTION

Age-related decline in cognitive and sensorimotor functions in older adults has been linked with changes in the brain's structural and functional connectivity patterns (Seidler et al., 2010; Damoiseaux, 2017). These age-related differences in functional connectivity that mediate information flow across the brain have been attributed in part to the decline in white matter integrity in older adults (Wu and Hallett, 2005; Zahr et al., 2009; Sullivan et al., 2010; Bruijn, 2014). Additionally, mounting evidence from neuroimaging suggests age-related changes in cortico-cortical connectivity in the motor network of healthy older adults contribute to age-related declines in sensorimotor functions. Functional cortico-cortical connectivity measures derived from dual-site transcranial magnetic stimulation (dsTMS) in healthy older adults also have shown reduced facilitatory and inhibitory inputs from secondary motor areas, including the supplementary motor area (SMA) (Green et al., 2018) and dorsal premotor cortex (PMd) (Ni et al., 2014), to primary motor cortex (M1). Posterior parietal cortex (PPC), a region involved in transforming sensory information into motor commands (Crawford et al., 2003, 2004; Andersen and Cui, 2009), is strongly interconnected with motor areas through white-matter tracts of the superior longitudinal fasciculus (Makris, 2004). These reciprocal glutamatergic parietal-frontal circuits are likely excitatory (Tokuno and Nambu, 2000; Dum and Strick, 2002; Matsumoto et al., 2006) and underlie control processes for skilled voluntary movements such as dexterous finger movements

required during the manipulation of objects (Filimon, 2010; Davare et al., 2011; Vesia and Crawford, 2012; Turella and Lingnau, 2014; Gallivan and Culham, 2015).

Human neuroimaging studies have implicated parieto-frontal brain regions in sensorimotor control of human hand behavior (Gallivan et al., 2011, 2013; Fabbri et al., 2014; Monaco et al., 2020; Turella et al., 2020). Anatomical findings in non-human primates have shown direct monosynaptic inputs to M1 from PPC in the control of hand movements (Strick and Kim, 1978; Rozzi et al., 2005; Bruni, 2018). A similar direct functional and anatomical parieto-motor pathway has been seen in human imaging (Koch et al., 2010). A number of dsTMS findings also have shown direct facilitatory parieto-motor connectivity in both the resting and active brain for hand actions in young adults (Koch et al., 2007, 2008; Ziluk et al., 2010; Cattaneo and Barchiesi, 2011; Karabanov et al., 2013; Vesia et al., 2013, 2017). Similarly, recent findings from intraoperative dual cortical stimulation in humans have provided direct evidence that the inferior parietal lobule exerts short-latency excitatory effects on cortical motor output (Cattaneo et al., 2020). Importantly, a recent neuroimaging study points to reduced coupling of parietal and premotor areas as a possible mechanism for the decreased perceptual motor speed observed in older adults (Michely et al., 2018). A question that remains, however, is whether the well-established age-related decline in sensorimotor performance relates to age-related differences in parieto-motor connectivity in older adults. We used dsTMS to characterize patterns of functional PPC-M1 connectivity and a standard pegboard test to estimate skilled motor performance in young and older adults. We hypothesized that facilitatory connectivity between PPC and M1 is reduced in older adults.

METHODS

Participants

Thirteen young adults (YA, 8 females, 19.9 ± 1.3 years) and thirteen older adults (OA, 5 females, 72.2 ± 5.5 years) provided written consent to participate in the study. All participants were right-handed as assessed by the Edinburgh Handedness Inventory (Oldfield, 1971). All participants were screened for any contraindications to TMS (Keel et al., 2001; Rossi et al., 2011) and had no history of neurological disorders. To assess weekly frequency and duration of various physical activities undertaken by older adults, we administered the Community Health Activities Model Program for Seniors self-report questionnaire (CHAMPS), which revealed that all were very physically active (total caloric expenditure per week: 3,799.5 ± 869.6; (Stewart et al., 2001). Cognitive function was assessed in the older adults using the Montreal Cognitive Assessment (MoCA score, ≥26) (Nasreddine et al., 2005) and Mini-Mental State Exam (MMSE score ≥27) (Folstein et al., 1975). Those who took CNS-active medications within 48 h of the study were excluded. All procedures were approved by the University of Michigan Institutional Review Board (HUM00155459) in accordance with the Declaration of Helsinki.

Procedures

Transcranial magnetic stimulation in a conditioning-test approach with two coils (Lafleur et al., 2016; Hallett et al., 2017; Goldenkoff et al., 2020) was used to measure connectivity between left PPC and left M1 (**Figure 1A**). A test stimulus (TS) was delivered to M1 with a figure-8 coil (D70^2, 7 cm diameter) connected to a Magstim 200^2 stimulator (Magstim, Whitland, UK) with a monophasic waveform. The TS coil was held tangential to the skull at 45° from the mid-sagittal line, inducing a current in the posterior-anterior direction in the underlying cortical tissue. TS intensity was set to produce a motor evoked potential (MEP) of ∼1 mV in the first dorsal interosseous muscle or the abductor pollicis brevis muscle in the right hand (Rossini et al., 2015). Left M1 was defined as the optimal scalp position for coil placement where stimulation evoked the largest MEP from the quiescent right-hand target muscle. PPC stimulation was applied to the P3 electrode position of the international 10-20 electroencephalogram (EEG) coordinate system using commercially available EEG head caps in each participant. The site is situated over angular gyrus (BA 39) of the inferior parietal lobule (Herwig et al., 2004; Okamoto et al., 2004) and corresponds with activation foci for hand actions identified by neuroimaging (Vesia and Crawford, 2012). A conditioning stimulus (CS) was delivered to PPC with another figure-8 coil (D50 Alpha B.I., 5 cm diameter) connected to a Magstim 200^2 stimulator (Magstim, Whitland, UK) with a monophasic waveform and a posterior-anterior current direction. The CS coil was held tangential to the skull at 90° from the mid-sagittal line. CS preceded TS by an inter-stimulus interval (ISI) of 4, 6, or 8 ms. PPC stimulation intensity was applied at 70, 90, 110, and 130% of resting motor threshold (RMT), similar to previous work (Koch et al., 2007). RMT was defined as the lowest intensity that evoked MEPs of at least 50 μV in peak-to-peak amplitude in three of five consecutive trials with the PPC coil from the right-hand muscle (Rossini et al., 1994). Each stimulus-response curve was repeated for each ISI. Twelve single-pulse stimuli (TS alone) to M1 and paired-pulse stimuli (CS-TS) at each PPC stimulation intensity were delivered in random order within an experimental block (60 trials) with both hands at rest. Stimuli were applied every 5 s. The order of the ISI block for each stimulus-response curve was counterbalanced across participants. A frameless stereotactic neuronavigation system (Brainsight; Rogue Research, Montreal, Canada) was used to ensure consistency in the TMS coil position throughout the stimulation session. After the electrophysiological measurements were completed, motor skill performance was examined by a test of hand dexterity, the Grooved Pegboard Test (GPT, Lafayette Instrument # 32025) using standard procedures (Wang et al., 2011).

Data Analysis

Electromyography (EMG) signals were recorded from the right-hand target muscle using bipolar surface electrodes (Model 2024F, Intronix Technologies Corporation), filtered (band-pass, 20 Hz to 2.5 kHz), and digitized at 5 kHz (Micro 1401 Cambridge Electronics Design). The peak-to-peak amplitude of the MEPs (mV) occurring between 15 and 100 ms after the

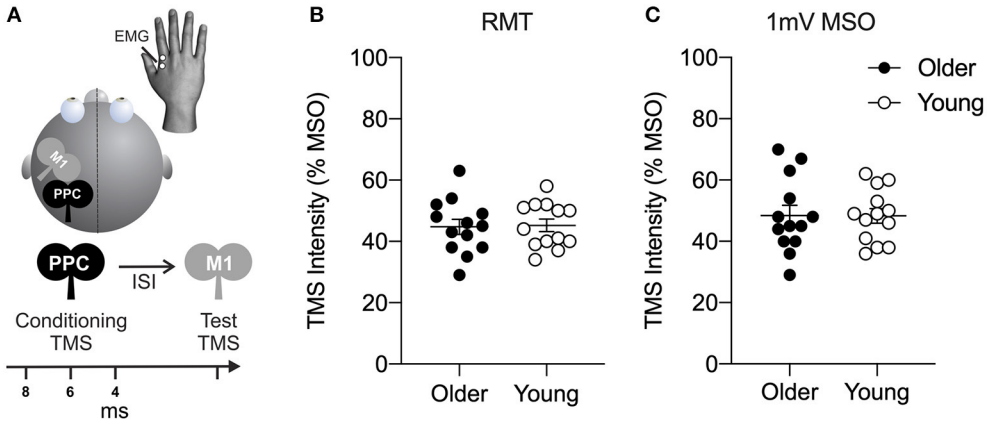

FIGURE 1 | (A) Dual-site transcranial magnetic stimulation in a conditioning-test approach was used to probe connectivity between left posterior parietal cortex (PPC) and ipsilateral primary motor cortex (M1). The conditioning stimulus (CS) was delivered at an intensity of 70, 90, 110, or 130% of resting motor threshold (RMT) to the PPC. CS was delivered 4, 6, or 8 ms prior to a test stimulus (TS) delivered to primary motor cortex (M1). Resulting motor evoked potential (MEP) amplitudes were recorded using electromyography (EMG) in the right-hand target muscle at rest. **(B)** Motor excitability plots showing transcranial magnetic stimulation (TMS) intensity (expressed as a percentage of the maximum stimulator output, MSO) of resting motor threshold (RMT) and **(C)** TMS intensity to elicit a motor evoked potential (MEP) of 1 mV for older (filled circles) and young (open circles) adults. Mean and SE are presented.

TS were measured for each trial. Trials in which test pulses coincided with motor activity or failed to elicit reliable MEPs (i.e., value exceeded 1.5 times the interquartile range for the participant) were removed from the analysis (∼2% of trials). The mean MEP amplitude for paired-pulse stimulation (CS-TS) was normalized by calculating the ratio of the amplitude relative to the mean single-pulse TS alone to M1 for each participant. Separate split-plot analysis of variances (ANOVAs) were carried out on the normalized MEP amplitudes for each PPC stimulus-response curve at each PPC stimulation intensity using Age (two levels: young or older adults) as a between-subjects factor and ISI (three levels: 4, 6, or 8 ms) as a within-subjects factor. The Bonferroni method was used for *post-hoc* *t*-test comparisons. The Greenhouse-Geiser method was used to correct for sphericity. Independent sample *t* -test was used to compare motor excitability and behavioral measures between groups. Paired *t*-tests also were conducted on the absolute amplitudes of the test MEP and conditioned MEP for the PPC stimulation intensity of 90% RMT at 6 ms ISI to evaluate facilitation and inhibition within the older and young adults. Correlations between neurophysiological and behavioral data were tested with Pearson's coefficient. Statistical analysis was performed using IBM-SPSS Statistics Version 26. A significance threshold was set at $p < 0.05$. Partial η squared (η_p^2) values were computed as a measure of effect size. Cutoffs for effect sizes are considered small (≥0.01), medium (≥0.06), and large (≥0.14) (Cohen, 1992). Mean and standard error values are reported.

RESULTS

As shown in **Figure 1**, no significant age difference was found in measures of motor excitability (all $t_{24} < 0.71$, all $p > 0.49$). The RMT of maximum stimulator output (MSO) was 45.2 ± 2.0% MSO for young adults and 44.8 ± 2.5% MSO for older adults (**Figure 1B**). The intensity to elicit a MEP amplitude of 1 mV in the right-hand target muscle was 48.3 ± 2.4% MSO for young adults and 48.4 ± 3.7% MSO for older adults (**Figure 1C**).

Figure 2A shows PPC-M1 connectivity in young and older adults. Facilitation seen in the MEP amplitude ratio in young adults was reduced in older adults with PPC stimulation at 90% RMT for the ISI of 6 ms (significant Age and ISI interaction: $F_{(1.96,47.0)} = 3.4$, $p = 0.043$, $\eta_p^2 = 0.12$; no main effect of Age: $F_{(1,24)}1.17$, $p = 0.29$, $\eta_p^2 = 0.05$; no main effect of ISI: $F_{(1.96,47.0)} = 0.34$, $p = 0.71$, $\eta_p^2 = 0.01$). Specifically, *post hoc* tests confirmed that the ratio of the MEP amplitude was significantly different between the age groups for the PPC stimulus-response curve at 90% RMT at the 6 ms ISI (Bonferroni's *t*-test: $t_{22.9} = 2.84$, $p = 0.028$). The results show that the facilitation between left PPC and ipsilateral M1 connections in young adults is reduced in older adults. Closer inspection of the individual normalized data confirmed that left PPC stimulation intensity of 90% RMT at 6 ms ISI caused inhibition of corticospinal excitability in ipsilateral M1 in about 69% of the older adults (**Figure 2B**). A similar pattern emerged at this timing and intensity when comparing the absolute amplitude of the conditioned and test MEP (**Figure 2C**). Paired *t*-tests revealed a trend toward significance in the facilitation of the conditioned MEP compared to TS alone in young adults ($t_{12} = 2.04$, $p = 0.06$), while the analysis within older adults did not reach significance ($t_{12} = 1.38$, $p = 0.19$). It is worth noting that single-pulse TS MEPs for the older adults were more variable than young adults, possibly influencing the normalized MEP amplitude ratio.

In a series of control experiments, we also verified whether the PPC-M1 connectivity at rest would differ with different PPC stimulation intensities. In each case, no significant age difference was found in the PPC stimulus-response curve at intensities of 70, 110, or 130% RMT (**Figure 3**). At 70% RMT (**Figure 3A**),

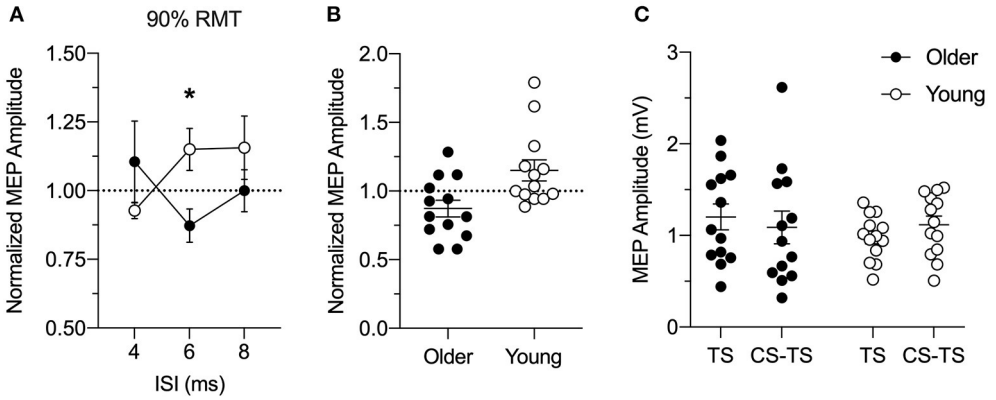

FIGURE 2 | (A) Group analysis of the effects of left posterior parietal cortex (PPC) stimulation at 90% resting motor threshold (RMT) intensity on motor evoked potential (MEP) amplitude induced by left M1 stimulation for older ($n = 13$; closed circles) and young ($n = 13$; open circles) adults at rest. The conditioning stimulus (CS) to PPC preceded a test stimulus (TS) to the primary motor cortex (M1) by an inter-stimulus interval (ISI) of 4, 6, or 8 ms. MEP amplitude was normalized as a ratio of the MEP amplitude evoked by paired-pulse stimulation (CS-TS) to that evoked by single-pulse stimulation (TS) to M1 alone (dashed line). Y = 1 indicates no effect of TMS to PPC on M1 excitability, whereas ratios higher than 1 indicate increased and ratios lower than 1 indicate decreased M1 excitability because of PPC stimulation. Facilitation in the ratio of MEP amplitude in young adults was reduced in older adults with PPC stimulation delivered at an intensity of 90% RMT at ISI of 6 ms. **(B)** Individual conditioned MEP amplitudes for the stimulus-response curve at 90% of RMT at the 6 ms ISI for older ($n = 13$, filled circles) and young ($n = 13$, open circles) adults normalized to TS alone (dashed line). **(C)** Individual absolute values of MEP amplitudes (mV) for the single-pulse TS to M1 and paired-pulse CS-TS to PPC at an intensity of 90% RMT at the 6 ms ISI for older (filled circles) and young (open circles) adults. Mean and SE are presented. $^*p < 0.05$.

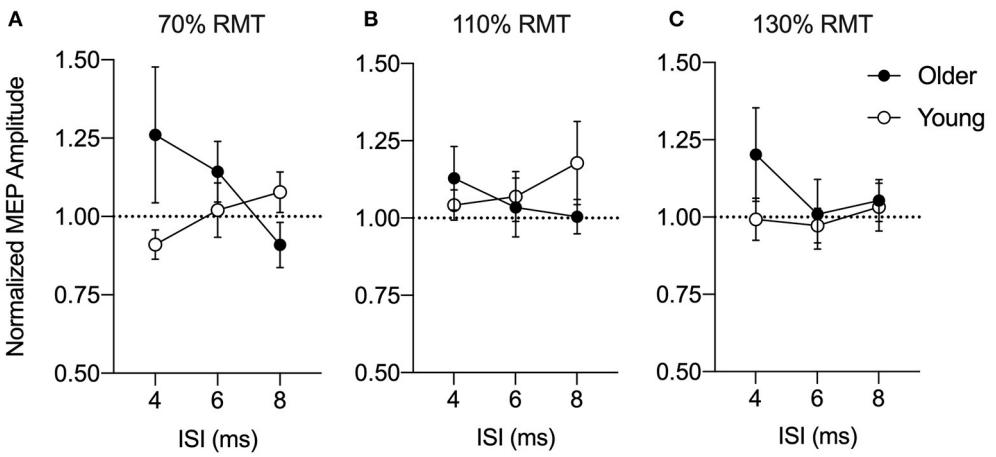

FIGURE 3 | Group-averaged conditioned motor evoked potential (MEP) amplitudes for a posterior parietal cortex (PPC) stimulus-response curve at 70% **(A)**, 110% **(B)**, and 130% **(C)** of resting motor threshold (RMT) at an inter-stimulus interval (ISI) of 4, 6, or 8 ms normalized to a test stimulus (TS) alone (dashed line). MEP amplitudes are expressed as a ratio of the amplitude relative to the mean single-pulse TS alone to the primary motor cortex (M1) for older ($n = 13$, filled circles) and young adults ($n = 13$, open circles). Mean and SE are presented.

a split-plot ANOVA showed that there was no significant effect of Age [$F_{(1,24)} = 1.42$, $p = 0.25$, $\eta_p^2 = 0.06$], ISI ($F_{(1.44,34.6)} = 0.40$, $p = 0.60$, $\eta_p^2 = 0.02$), nor an interaction effect [$F_{(1.44,34.6)} = 2.52$, $p = 0.11$, $\eta_p^2 = 0.10$]. At 110% RMT (**Figure 3B**), a split-plot ANOVA showed that there was no significant effect of Age [$F_{(1,24)} = 0.28$, $p = 0.60$, $\eta_p^2 = 0.01$], ISI [$F_{(1.73,41.5)} = 0.11$, $p = 0.90$, $\eta_p^2 = 0.005$], nor an interaction effect [$F_{(1.73,41.5)} = 1.05$, $p = 0.35$, $\eta_p^2 = 0.04$]. Similarly, at 130% RMT (**Figure 3C**), a split-plot ANOVA showed that there was no significant effect of Age [$F_{(1,24)} = 1.32$, $p = 0.26$, $\eta_p^2 = 0.05$], ISI [$F_{(1.93,46.29)} = 0.64$, $p = 0.53$, $\eta_p^2 = 0.03$], nor an interaction effect [$F_{(1.93,46.29)} = 0.61$, $p = 0.54$, $\eta_p^2 = 0.03$].

As shown in **Figure 4A**, young adults were significantly faster than older adults at completing the GPT (young adults = 68.0 ± 8.6 s vs. older adults = 90.7 ± 22.2 s, $t_{24} = 3.44$, $p = 0.003$). **Figure 4B** shows associations between the PPC-M1 connectivity at rest and motor skill performance in young and older adults. A correlation analysis for each age group showed that the normalized MEP amplitude for the PPC stimulation intensity of 90% RMT at the 6 ms ISI did not correlate with GPT completion

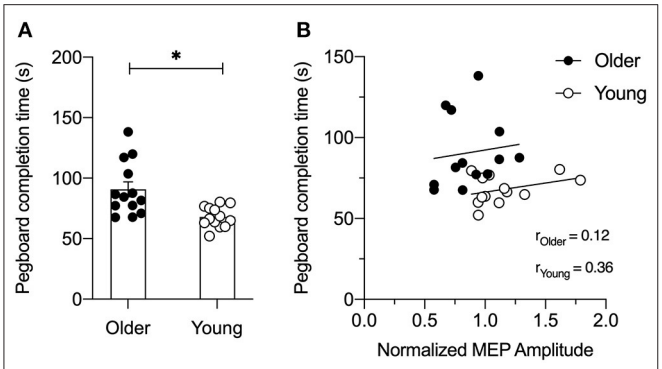

FIGURE 4 | (A) Bar graph for group averaged completion times for the Grooved Pegboard Test in seconds (s). Individual data are presented for older (filled circles) and young (open circles) adults. As expected, young adults performed the task faster compared to older adults. Error bars represent SE. $^*p < 0.05$. **(B)** Correlation between normalized motor evoked potential (MEP) at the 6 ms inter-stimulus interval (ISI) for PPC stimulus-response curve at 90% resting motor threshold (RMT) and Grooved Pegboard Test completion time for each older (filled circles) and young adult (open circles). A simple linear regression line is superimposed over the individual data points for each group. Normalized MEP amplitude did not correlate with the Grooved Pegboard Test performance in both young and older adults (*all p's* > 0.2).

time in both young ($r = 0.130$; $p = 0.23$) and older adults ($r = 0.015$; $p = 0.69$).

DISCUSSION

A reduction of facilitation in a parieto-motor connection responsible for skilled hand movements was demonstrated in older adults compared to young adults. Using a dsTMS approach, we found that a conditioning stimulus to PPC with a 90% RMT intensity delivered 6 ms prior to a test stimulus to M1 resulted in a facilitation of the MEP amplitude in young but not older adults. In fact, the PPC-M1 interaction in the older group changed from facilitation to inhibition. The present findings are in line with prior dsTMS work in young adults showing short-latency PPC-M1 functional interactions are selectively facilitated at rest when applying CS to PPC at an intensity of 90% RMT (Koch et al., 2007; Karabanov et al., 2013). In the current study, the reduced PPC-M1 facilitation in older adults may be related to impairments of high-level movement planning signals in PPC as demonstrated by their slowed completion time of the GPT (Andersen and Cui, 2009). However, future work will need to better characterize the relationship between functional PPC-M1 connectivity and skilled motor performance. For instance, previous dsTMS studies have shown that functional PPC-M1 connectivity is modulated early in the motor plan for different types of hand actions at a similar ISI and conditioning stimulation intensity in young adults (Koch et al., 2008; Vesia et al., 2013, 2017). Yet, no work has investigated whether there is a relationship between functional PPC-M1 interactions during grasp preparation and manual dexterity in older adults. Future investigation is needed to determine whether similar age-related differences occur during these action-associated processes. It also should be noted that it remains unclear whether the non-significant association between reduced facilitation of parieto-motor functional connections and manual dexterity in older adults is present in a larger sample size given the moderate sample size in the current study. One possible explanation of the current results is that widespread motor plans involving multiple, parallel parieto-premotor-motor circuits could modulate corticospinal output associated with sensorimotor hand control (Koch and Rothwell, 2009; Davare et al., 2010, 2011; Vesia and Davare, 2011; Turella and Lingnau, 2014; Vesia et al., 2018). It also is possible that the relationship between cortico-cortical connections in the motor system and skilled hand behavior likely encompasses a much broader range of brain regions within frontal, parietal, and temporal cortices to support the flexibility of human hand behavior (Grafton, 2010; Gallivan and Culham, 2015; Monaco et al., 2020; Turella et al., 2020).

This interpretation is in line with prior dsTMS studies demonstrating age-related decline between M1 and frontal areas in the motor cortical network such as dorsolateral prefrontal cortex (Fujiyama et al., 2016), SMA (Green et al., 2018), and PMd (Ni et al., 2014). Together, these findings suggest an age-related reduction in functional connectivity from action-associated cortical areas to M1 in older adults. It is possible that the degraded facilitatory inputs from PPC to M1 in older adults likely represent an early marker of age-related decline for functional connectivity underlying complex motor skills. This view is in line with theoretical suggestions that older adults recruit frontal cortical areas to compensate for bottom-up sensory processing in posterior cortical areas when performing more cognitive-demanding motor tasks (Davis et al., 2008). Indeed, a large body of research provides complementary evidence linking these age-related differences in functional activation and connectivity patterns with cognitive and motor performance (see reviews Seidler et al., 2010; Damoiseaux, 2017). Functional neuroimaging studies have consistently demonstrated enhanced prefrontal influences on the motor system in response to increased task demands in older adults (Heuninckx et al., 2005; Wu and Hallett, 2005; Cabeza et al., 2018). One such study found that prefrontal areas compensated for decreased parietal influences on premotor areas associated with a decline in perceptual motor speed with advancing age (Michely et al., 2018). This is also consistent with evidence linking age-related decline in cognitively demanding motor tasks with structural changes in white matter tracts that connect sensorimotor, frontal, and parietal regions in older adults (Stewart et al., 2014). The reduced efficacy of this connection is also supported by findings in individuals with Parkinson's disease (Palomar et al., 2013) and stroke (Schulz et al., 2015) that show a more favorable motor outcome related to higher levels of communication between frontal and parietal areas in the motor system. Additionally, dsTMS evidence has shown that this selective age-related decrease in PPC-M1 facilitation in healthy older adults is exacerbated at early clinical stages of Alzheimer's disease (Bonnì et al., 2012). This decreased functional connectivity precedes disease-related changes in cognitive-related frontal areas and could in part represent a key driver of cognitive decline in Alzheimer's disease (Koch et al., 2019).

It is worth noting that neurobiological aging is a complex process involving interactions between local cortical and brain network plasticity (Freitas et al., 2013). In the case of

aging, compensatory processes likely depend on the neural reorganization and recruitment of alternative circuits to attenuate cognitive and motor declines that occur with age (Cabeza et al., 2018). Future longitudinal studies delineating the specific contribution of PPC on motor related frontal circuits combining TMS with neuroimaging approaches will be needed to gain insight into the mechanisms of system-level plasticity across the lifespan. We recognize that age-related neural decline such as brain atrophy, synaptic loss, and white matter degradation could account for the observed MEP amplitude differences between young and older adults (Giorgio et al., 2010; Farokhian et al., 2017). However, we believe a global effect on mechanisms underlying aging is unlikely to account for the highly specific reduction in PPC-M1 facilitation in the current study. A methodological limitation of the study is that we did not selectively localize PPC sites for hand actions at the individual level using fMRI or task-based dsTMS. Prior dsTMS work has shown anatomical and functional differences in connectivity between PPC regions and M1 (Karabanov et al., 2013). This raises the possibility that the effects at other ISIs and conditioning stimulation intensities in our study could be dependent on different neural substrates. Further studies will need to examine the effect of nearby parietal regions on motor excitability to account for the individual differences in functional connectivity among older adults. Finally, we recognize that the older adults tested in the current study were high-functioning and in relatively good health based on self-reports. Therefore, future work is needed with a more heterogenous subset of older participants to clarify the effects of physical activity and other environmental factors on age-related cognitive and motor deficits.

We conclude that the reduced PPC-M1 facilitation in older adults may be an early marker of age-related decline in the neural control of movement. Our findings could have implications for understanding functional parieto-frontal connectivity affected by advancing age in both healthy and clinical populations. Importantly, dsTMS methods could be used to develop better diagnostic tools and treatment approaches (Fox et al., 2012, Hallett et al., 2017; Goldenkoff et al., 2020). We propose that prospective strengthening of PPC-M1 circuitry in healthy adults might be a fruitful therapeutic path to counteract the gradual age-related breakdown in functional connectivity within the motor-related network associated with motor impairments. It is possible that the preservation of these neural substrates could enhance resilience of the intact circuitry and minimize compensatory shifts in brain networks that maintain optimal cognitive and motor performance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Michigan Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EG: methodology, formal analysis, investigation, writing—original draft, writing—review & editing, visualization, and project administration. RL: methodology, investigation, project administration, formal analysis, and writing—review & editing. SB: methodology, resources, writing—review & editing, and supervision. MV: conceptualization, methodology, resources, writing—review & editing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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The 4-(Phenylsulfanyl) butan-2-one Improves Impaired Fear Memory Retrieval and Reduces Excessive Inflammatory Response in Triple Transgenic Alzheimer's Disease Mice

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Alzheimer's disease (AD) is a neurodegenerative disease characterized by an excessive inflammatory response and impaired memory retrieval, including spatial memory, recognition memory, and emotional memory. Acquisition and retrieval of fear memory help one avoid dangers and natural threats. Thus, it is crucial for survival. AD patients with impaired retrieval of fear memory are vulnerable to dangerous conditions. Excessive expression of inflammatory markers is known to impede synaptic transmission and reduce the efficiency of memory retrieval. In wild-type mice, reducing inflammation response can improve fear memory retrieval; however, this effect of this approach is not yet investigated in 3xTg-AD model mice. To date, no satisfactory drug or treatment can attenuate the symptoms of AD despite numerous efforts. In the past few years, the direction of therapeutic drug development for AD has been shifted to natural compounds with anti-inflammatory effect. In the present study, we demonstrate that the compound 4-(phenylsulfanyl) butan-2-one (4-PSB-2) is effective in enhancing fear memory retrieval of wild-type and 3xTg-AD mice by reducing the expression of TNF-α, COX-2, and iNOS. We also found that 4-PSB-2 helps increase dendritic spine density, postsynaptic density protein-95 (PSD-95) expression, and long-term potentiation (LTP) in the hippocampus of 3xTg-AD mice. Our study indicates that 4-PSB-2 may be developed as a promising therapeutic compound for treating fear memory impairment of AD patients.

Keywords: Alzheimer's disease, inflammation, 3xTg-AD, fear conditioning, 4-(phenylsulfanyl) butan-2-one, natural compound, hippocampus, CA3

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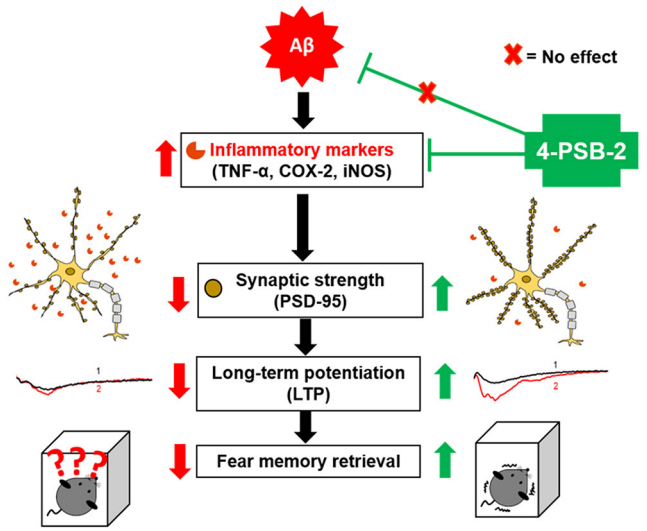
INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder known to involve neuronal inflammation (Newcombe et al., 2018) and impaired memory retrieval, including episodic memory, recognition memory, spatial memory (Serrano-Pozo et al., 2011; Wahl et al., 2017), and fearful and traumatic memory (Hamann et al., 2002). Retrieval of fear memory elicits a fear response that helps avoid



Graphical Abstract | Schematic abstract. We are the first to report that 4-PSB-2 acts as memory enhancer and anti-inflammatory compound, which can reverse impairments in fear memory retrieval in 3xTg-AD mice without changing Aβ levels. 4-PSB-2 reduces the expression of several inflammatory markers including TNF-α, COX-2, and iNOS, increases PSD-95 expression in the hippocampus, and improves synaptic dysfunction in 3xTg-AD mice, which may account for its effect in improving fear memory retrieval.

predators and natural threats. Thus, the fear response is important for survival, and the underlying neural circuits are highly conserved across species (Wotjak and Pape, 2013). Studies of AD patients have revealed that the fear response is defective at the onset stage of AD (Nasrouei et al., 2020). The AD model animals also exhibit impaired contextual fear memory (Billings et al., 2005; Kishimoto et al., 2017). An excessive inflammatory response is known to associate with impairments of working memory, remote memory stabilization, and spatial memory in AD (Murray et al., 2012; Wang et al., 2014; Mariani et al., 2017; Scuderi et al., 2018); however, its relationship with defective fear memory of AD is not clear yet.

The inflammatory response in the brain has been implicated in the initiation and progression of the pathogenesis of AD (Newcombe et al., 2018). The two AD pathological hallmarks, extracellular amyloid-beta (Aβ) deposition and intracellular neurofibrillary tangles, are known to increase inflammatory response and impede several types of memory functions, including episodic memory, recognition memory, semantic memory, spatial memory, and emotional memory (Serrano-Pozo et al., 2011; Klein-Koerkamp et al., 2012; Wahl et al., 2017). Early accumulation of Aβ and tau tangles can also induce astrogliosis and microglial activation, resulting in the elevated expression of proinflammatory mediators, including interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), and interleukin-6 (IL-6) (Wang et al., 2015). Increases in these proinflammatory mediators can cause neurotoxicity, loss of synaptic transmission, reduced long-term potentiation (LTP) induction, and impaired cognitive functions in AD (Garwood et al., 2011; Fakhoury, 2018; Rajendran and Paolicelli, 2018). Previous studies have demonstrated that fear memory retrieval deficit is related to the decrease of LTP induction in AD mouse models such as Tg2576 mice (Comery et al., 2005), APP/PS1 mice (Gu et al., 2016), and 5XFAD mice (Kimura and Ohno, 2009). To date, no drug or treatment can cure this disease or effectively reduce the symptoms in humans. Most clinical trials of drugs targeting the clearance of Aβ have had limited success and failed to rescue memory functions (Morrison, 2016), while reducing inflammatory responses seem to delay the onset of AD symptoms (Akiyama et al., 2000; Businaro et al., 2018). The nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to prevent or delay the onset of AD (Zhang et al., 2018). However, it was not effective and caused various adverse side effects including gastrointestinal bleeding, hypertension, and nephrotoxicity in elderly patients (Wongrakpanich et al., 2018). Therefore, finding a compound that can effectively prevent or treat fear memory impairment and reduce (excessive inflammatory response) in AD with less side effect is in an urgent need.

The compound 4-PSB-2 initially extracted from the soft coral *Cladiella australis*, has been shown to have anti-melanogenic effects via the suppression of tyrosinase activity in zebrafish embryos (Wu et al., 2015), and anti-inflammatory/neuroprotective effects with single-dose injection into a rat optic nerve crush model accompanied by reduced iNOS and cyclooxygenase-2 (COX-2) expression levels (Chien et al., 2016). Our recent study shows that $A\beta_{1-42}$ oligomers-induced elevation of inflammatory markers in retinal pigment epithelial cells can be attenuated with a single-dose application of 4-PSB-2. Administration of this compound significantly suppressed expressions of TNF-α, COX-2, and iNOS (Varinthra et al., 2020). Besides, 4-PSB-2 is lipophilic; therefore, it is suitable as a drug candidate. According to all the 4-PSB-2 characteristics, we chose it to investigate whether reducing inflammation response in AD mice can improve impaired retrieval of fear memory. We injected the 4-PSB-2 into wild type and 3xTg-AD mice before fear memory testing. We found that this compound can effectively improve the deficit of contextual fear memory retrieval of the 3xTg-AD mice, reduce neuroinflammation response, and increase synaptic plasticity in the hippocampus.

METHODS

Ethics Statement

All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Tzu Chi University (TCU, #108030), Taiwan and followed the guidelines of the Taiwan Ministry of Science and Technology on the ethical treatment of animals.

Animals

Six-month-old male wild-type C57BL/6 mice with some mixed SV129 genetic markers, initially provided by the National Laboratory Animal Centre (Taiwan), were purchased and maintained undisturbed in the Laboratory Animal Centre of Tzu Chi University. Six-month-old 3xTg-AD mice were provided by Hei-Jen Huang from the Department of Nursing, Mackay Junior

College of Medicine, Nursing and Management (Taiwan), and Hsiu Mei Hsieh-Li from the Department of Life Science, National Taiwan Normal University (Taiwan). The 3xTg-AD mice, initially purchased from the Jackson Laboratory (Stock #34830 JAX), harbor three mutations associated with AD and dementia in the genetic background of C57BL/6;129. The three mutations are two familial AD mutations (APP Swedish and PSEN1 M146V), and one mutation associated with frontotemporal dementia and parkinsonism-17 (tau P301L). Six-month-old male 3xTg-AD mice were used in this study. All mice were housed in individual plastic and metal cages in a temperature-controlled room with a 12-h light/dark cycle until the behavioral tasks were performed. Food and water were provided ad libitum.

Preparation of 4-PSB-2 Solution and Treatment

4-PSB-2 was provided by the Research Center of National Research Program for Biopharmaceuticals, Taiwan and its structure is shown in **Figure 1A** (Wen et al., 2014). 4-PSB-2 was dissolved in saline/DMSO at the ratio of 9:1. Three different concentrations (5, 10, and 15 mg/kg) of 4-PSB-2 were used in this study. 4-PSB-2 was intraperitoneally injected into mice immediately after trace fear conditioning (TFC).

Trace Fear Conditioning

Wild-type (WT) mice were divided into two groups: (1) the WT group that did not undergo TFC and (2) the WT group that underwent TFC. Each group contained 3 subgroups (n = 5/subgroup), which included mice injected with (1) saline, (2) saline + dimethyl sulfoxide (DMSO), or (3) 15 mg/kg 4-PSB-2. 3xTg-AD mice (n = 8–11/group) were divided into three groups: (1) the untreated 3xTg-AD group, (2) sham 3xTg-AD group (saline + DMSO), and (3) the 3xTg-AD group treated with 15 mg/kg of 4-PSB-2. Fear conditioning was performed as described previously (Pai et al., 2018). On days 1-3, WT mice (n = 5/group) and 3xTg-AD mice were habituated to the conditioning chamber for 15 min per day. On day 4 (TFC day), the mice were placed in the chamber for 2 min and exposed to three trials of a tone (6,000 Hz, 85 dB; conditional stimulus) for 20 s followed by a 10-s interval and a 1-s foot shock (2 mA; unconditional stimulus). 4-PSB-2 was injected into mice immediately after TFC. Twenty-four hours later, the mice were placed in the same conditioning chamber for 6 min without the tone or foot shock for the contextual test. One hour later, the tone test was performed. The mice were placed in a new chamber for 1 min without a tone or foot shock and then exposed to a tone (6,000 Hz, 85 dB) for 6 min. Freezing behaviors were recorded and analyzed by FreezeScan software (CleverSys, Inc., VA, USA), and the freezing percentage was calculated as (total freezing time/total test time) × 100. Freezing behavior was characterized by the inhibition and absence of movement, heavy breathing, and minimal movement for normal respiration. Head scanning and sleeping were not considered freezing behaviors.

Electrophysiological Recording

After TFC, the WT and 3xTg-AD mice were anesthetized and immediately sacrificed followed by taking the whole brain to incubate in ice-cold artificial cerebrospinal fluid (ACSF). Then, the hippocampus was horizontally sectioned for about 350 μm thickness by a vibrating microtome (Leica VT1000 S, Leica Biosystems Inc., Nussloch, Germany) in oxygenated (95% O_2/5% CO_2) ACSF. The hippocampal slices were incubated at 26°C for 2 h before recording. To induce LTP, the concentric bipolar tungsten electrodes and the recording glass pipettes with a micropipette puller were placed in the Schaffer collateral–commissural fibers at the stratum radiatum of the hippocampal CA1 region. During LTP inducing and recording, the slices were perfused continuously with ACSF at a speed of 20 rpm. The stimulation intensity was adjusted between 0 and 10 V for each slice. The field excitatory postsynaptic potentials (fEPSP) were elicited to approximately 50% of the maximal response. Before LTP induction, a steady baseline was recorded every 20 s for 20 min. LTP was evoked by high-frequency stimulation (HFS) for 3 trials of 100 Hz with a 20 s interval between each trial for 60 s. Then, fEPSPs were stimulated every 20 s for 60 min. The signals were amplified by an Axon Multiclamp 700B amplifier (Axon Instruments, Foster City, CA), acquired at 10 kHz by an Axon Digidata 1550B plus HumSilencer (Axon Instruments, Foster City, CA) and filtered at 1 kHz. The slope of fEPSPs is measured using Axon pCLAMP 11 electrophysiology data acquisition and analysis software.

Western Blot Analysis

The mice were sacrificed by decapitation immediately after TFC to collect the hippocampi. The hippocampi were then homogenized in an ice-cold RIPA lysis buffer containing phosphatase and protease inhibitors (F. Hoffmann-La Roche AG, Basel, Switzerland). The samples were sonicated and centrifuged for 15 min at 13,500 × g at 4°C. The supernatants were collected, and the protein concentration was measured with the Bradford protein assay (Bio-Rad Laboratories, USA). Equal amounts of proteins from hippocampal tissues were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: mouse anti-PSD-95 (1:2,000, MA1-045, Thermo Fisher Scientific) and mouse anti-β-actin (1:10,000, A5441, Sigma-Aldrich). After that, the membranes were washed three times with 1X phosphate-buffered saline (PBS) containing 0.1% Tween-20 and incubated with a horseradish peroxidase-conjugated (HRP) anti-mouse antibody (1:10,000, 7076, cell signaling technology) for 1 h at room temperature. The proteins of specific molecular weights were visualized using enhanced chemiluminescence reagents (Western Lightning® Plus-ECL, PerkinElmer, MA, USA) and detected by a UVP BioSpectrum 810 imaging system. Band intensity was quantified using ImageJ software (downloaded from National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/download.html).

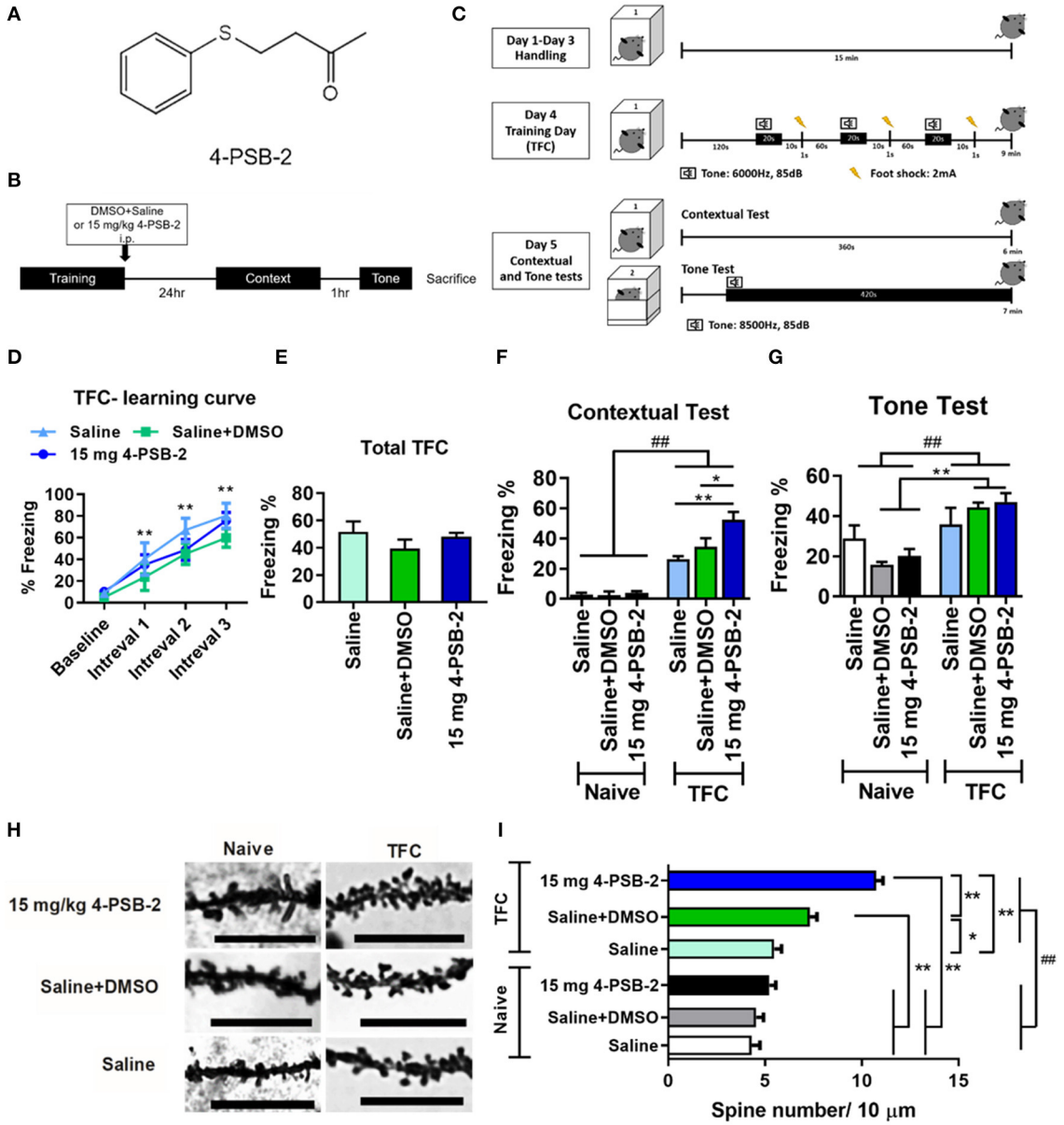


FIGURE 1 | 4-PSB-2 increased fear memory retrieval and dendritic spine density in the hippocampi of wild type (WT) mice. **(A)** The chemical structure of 4-PSB-2. **(B)** The timeline of 4-PSB-2 injection and TFC training. The WT mice were subjected to trace fear conditioning (TFC) and then immediately treated with 4-PSB-2. Contextual fear memory was tested 24 h later, and then a tone memory test was performed after a 1-h interval. **(C)** TFC protocol. The WT mice were handled in the conditioning chamber for 3 days before TFC. On day 4, the WT mice received 3 trials of TFC. Twenty-four hours later, their freezing responses to context were tested by placing them into the same conditioning chamber for 6 min, and then the tone memory test was performed in a different chamber for 6 min after a 1-h interval. **(D)** The WT mice acquired TFC when compared the freezing percentage at intervals 1–3 with baseline performance. **(E)** The results revealed that all groups were not significantly different in freezing percentage of total TFC. **(F)** There was a statistically significant interaction between the effects of TFC and treatments on freezing percentage change of contextual test in WT mice. The 4-PSB-2 injection at a concentration of 15 mg/kg significantly increased the retrieval of contextual fear memory and **(G)** slightly enhanced memory retrieval of a tone. **(H,I)** Among the trace fear-conditioned mice, the WT mice injected with 4-PSB-2 showed a significant increase in apical dendritic spine density. The number of WT mice in each group for TFC is shown. Naïve: saline ($n = 4$), saline + DMSO ($n = 5$), 15 mg 4-PSB-2 ($n = 5$); TFC: saline ($n = 4$), saline + DMSO ($n = 5$), 15 mg 4-PSB-2 ($n = 5$). $n = 2$/group for Golgi-COX staining. The results are shown in **(D–G)** and **(I)** are plotted as the means ± SEMs and were statistically analyzed by mixed-design repeated-measures ANOVA followed by Bonferroni test for **(D)**; two-way ANOVA followed by Tukey's test for **(F,G,I)**, ## indicates $p \leq 0.001$ between the factors; one-way ANOVA followed by Tukey's test for **(E)**, *Indicates $p \leq 0.05$, and **Indicates $p \leq 0.001$ between the groups. Bar = 10 μm.

Immunohistochemical Staining and Image Analysis

The hippocampi were taken at bregma −1.28 to −2.92 mm, then fixed with 4% paraformaldehyde (PFA) overnight at room temperature. After post-fixation, the brains were soaked in 30% sucrose at 4°C and cut coronally with cryostat every 20 μm. And then, the slides were fixed in methanol for 5 min at 4°C and dried at room temperature. Next, they were blocked with 1x PBS containing 2% BSA and 0.3% Triton X-100 for 2 h at room temperature and incubated with primary antibody: rabbit anti-TNF-alpha (1:300, ab6671, Abcam), rabbit anti-COX-2 (1:300, 12282, cell signaling technology), rabbit anti-iNOS (1:300, PA1-036, Thermo Fisher scientific), mouse anti-MAP2 (1:200, ab11267, Abcam), goat anti-GFAP (1:200, ab53554, abcam), goat anti-IBA1 (1:200, NB100-1028, Novus Biologicals), and rabbit anti-A11 (1:200, AHB0052, Thermo Fisher scientific), at 4°C, overnight. Sections washed in 1x PBS containing 0.3% Triton X-100 three times for 10 min and then incubated with the secondary antibody: Alexa 594 or Alexa 488 goat anti-rabbit IgG or goat anti-mouse (1:300, A-11037, A-11034, A-11056, A-11003, Thermo Fisher scientific, USA) or Donkey anti-goat IgG (1:300, ab150129, Abcam) for 1 h at room temperature in darkness. Sections were then again washed with 1x PBS containing 0.3% Triton X-100 three times for 10 min each, dried in room temperature, mounted with Fluoromount™ aqueous mounting medium, coverslipped and examined under fluorescence microscopy with 40X and 100X objective lens (Nikon ECLIPSE Ni-E). For calculating the positive area percentage of each antibody, 3–5 fields (200 × 200 μm) from 3 to 4 sections per mouse were quantified using ImageJ software (downloaded from National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/download.html).

Golgi-Cox Staining for Dendritic Spine Staining and Density Measurement

Mice were perfused transcardially with 0.9% saline followed by 4% PFA. After that, the whole brain was collected and fixed in 4% PFA overnight at room temperature. Next, the brains were transferred to Golgi-Cox solution and performed the staining process as previously described (Gibb and Kolb, 1998) for 5 days at 32°C. Then, the brains were transferred to 30% sucrose solution. The brains were sectioned at a thickness of 60 μm using a vibratome, and 5 sections/brain were collected for examination. Each group consisted of two animals, for each brain, 5 sections were chosen and 6 neurons from each section (3 from left, 3 from the right hippocampus—including DG and CA1) were selected for spine analysis. Neurons with properly stained dendrites were selected and the second to sixth proximal branch of apical dendrites were taken for spine counting. Spines were counted manually using ImageJ (downloaded from National Institutes of Health, Bethesda, MD, USA, website: https://imagej.nih.gov/ij/download.html). Spine density was represented as the number of spines per 10 μm of dendrite for 30–60 dendritic segments per group.

Molecular Descriptor Calculation

The chemical structure of 4-PSB-2 was analyzed by using the Dragon 7.0 software suite (Kode Chemoinformatics, Pisa, Italy). Dragon 7.0 software is widely used to calculate molecular descriptors in scientific research (Kawczak et al., 2018), including molecular weight (MW); Moriguchi log P (*M*logP); hydrogen bond donors (HDs), and hydrogen bond acceptors (HAs), which are neighboring electronegative ions bearing a lone pair of electrons; the number of rotatable bonds (RBN); and total polar surface area (TPSA).

Statistical Analysis

The mean ± standard error of the mean (mean ± SEM) of the data from this study was calculated and are plotted. The freezing percentage of total TFC, contextual and tone tests, locomotor activity, western blotting, immunohistochemical staining, and electrophysiological recording data in 3xTg-AD mice and WT –untreated were analyzed by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. The freezing percentage of contextual and tone tests, and synaptic spine density data in WT mice were analyzed the interaction of 2 factors including TFC and treatments on freezing percentage by two-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. The freezing percentage of the TFC learning curve of WT and 3xTg-AD mice were analyzed by mixed-design repeated-measures ANOVA followed by Bonferroni *post-hoc* test for multiple comparisons. Statistical significance of the differences among the groups was established at a P-value < 0.05. All graphs were plotted with GraphPad Prism 8.0 software.

RESULTS

Chemical Structure Properties of 4-PSB-2

The compound 4-PSB-2 was initially extracted from a soft coral *Cladiella australis*, then modified and synthesized by the Development Center for Biotechnology (Taiwan), the National Research Program for Biopharmaceuticals support (CS-1-G-103-002). To investigate the chemical structure properties of 4-PSB-2 (**Figure 1A**) and its potential for use as a therapeutic agent, the Dragon 7.0 software suite was used. Descriptors were calculated based on Lipinski's rules and are listed in **Table 1**. Lipinski's rules are a set of indicators of a compound's oral bioavailability,

TABLE 1 | The calculated molecular descriptors of 4-PSB-2.

Molecular Descriptors	4-PSB-2
MW (g/mol)	180.29
RBN	4
*M*logP	2.723
HD	0
HA	1
TPSA (Å2)	42.37

MW, Molecular weight; RBN, number of rotatable bonds; MlogP, Moriguchi log P; HDs, Hydrogen bond donors; HAs, Hydrogen bond acceptors; TPSA, Total polar surface area.

including a MW < 500 g/mol, RBN < 10, *M*logP < 4.15, HD < 5, HA < 10, and TPSA < 140 Å2. From **Table 1**, it is clear that the 4-PSB-2 obeys Lipinski's rules and is likely to show acceptable oral bioavailability. In particular, 4-PSB-2 has low TPSA and high *M*logP, indicating that it is lipophilic; thus was chosen for use in this study.

4-PSB-2 Enhanced the Retrieval of Contextual Fear Memory and Increased Hippocampal Dendritic Spine Density in Wild-Type Mice

To investigate the effect of 4-PSB-2 on fear memory retrieval following TFC, mice were exposed to TFC training and then immediately treated with saline+DMSO or 4-PSB-2 (**Figure 1B**). Twenty-four hours later, a contextual fear memory was tested by returning the mice to the same conditioning chamber without any tone or foot shock. One hour after the contextual test, memory for the tone was tested by placing the mice in a different chamber and exposing them to tone cue for 6 min without delivering any electric footshock (**Figure 1C**). The percentage of freezing indicated fear memory retrieval of the context and tone. A higher freezing percentage indicated better memory retrieval. The WT mice acquired TFC when compared the freezing percentage at intervals 1–3 with baseline performance [$F_{(3,33)} = 38.767, p < 0.001$; **Figure 1D**]. The results revealed that all groups were not significantly different in freezing percentage of total TFC [$F_{(2,11)} = 1.168$, $p = 0.347$; **Figure 1E**]. There was a statistically significant interaction between the effects of TFC and treatments on freezing percentage change of contextual test in WT mice [$F_{(2,22)} = 6.136$, $p < 0.01$]. Administration of 15 mg/kg 4-PSB-2 significantly enhanced the fear memory retrieval to context in WT mice [$p < 0.001$; **Figure 1F**]. There was a statistically significant interaction between the effect of TFC [$F_{(1,22)} = 31.045$, $p < 0.001$] but not treatments [$F_{(2,22)} = 0.334$, $p = 0.72$], on freezing percentage change of tone test in WT mice. Besides, the compound slightly enhanced fear memory retrieval to tone, although the effect was not statistically significant. Whereas, Naïve treated with saline + DMSO, Naïve treated with 15 mg/kg 4-PSB-2, TFC treated with saline + DMSO, and TFC treated with 15 mg/kg 4-PSB-2 groups showed a significant difference in fear memory retrieval to tone ($p < 0.001$; **Figure 1G**). 4-PSB-2 administration did not cause any impairment of locomotor activity, sociability, or anxiety in WT mice (**Supplementary Material** and **Supplementary Figure 1**).

To further verify the effect of 4-PSB-2 on memory retrieval in WT mice at the cellular level, dendritic spine density in the hippocampal region was evaluated using Golgi-Cox staining. The results indicated that at a concentration of 15 mg/kg, 4-PSB-2 significantly increased the apical dendritic spine density in the hippocampi of WT mice (**Figure 1H**). There was a statistically significant interaction between the effects of TFC and treatments on dendritic spine density in WT mice [$F_{(2,283)} = 18.65$, $p < 0.001$]. Dendritic spine density of naïve mice was not changed after saline + DMSO and 4-PSB-2 treatments. After TFC, WT mice treated with saline + DMSO showed significantly increased dendritic spine density than naïve and the TFC + saline groups. Treatment with 4-PSB-2 at 15 mg/kg to WT mice after TFC significantly increased the dendritic spine density compared with naïve groups and TFC groups ($p < 0.001$; **Figure 1I**).

4-PSB-2 Injection Into 3xTg-AD Mice Rescued Impaired Fear Memory Retrieval to Context and Improved the Synaptic Dysfunction in 3xTg-AD Mice

Since 4-PSB-2 at 15 mg/kg significantly enhanced the retrieval of contextual memory in WT mice (**Figure 1F**), we further tested whether it rescues impaired fear contextual memory in 3xTg-AD mice at this dose. 3xTg-AD mice exhibited Aβ deposition within neuronal and glial cells in the neocortex by 4 months and in the CA1 subregion of the hippocampus by 6 months (Oddo et al., 2003). The animals demonstrated impaired contextual fear and spatial memory at approximately 6 months of age (Billings et al., 2005). We injected 4-PSB-2 into 6-month-old 3xTg-AD mice immediately after TFC training and measured its effect on memory. After administration, 4-PSB-2 did not cause any impairment of locomotor activity in 3xTg-AD mice [$F_{(3,34)} = 1.048, p = 0.384$; **Figure 2A**, $F_{(3,34)} = 1.09, p = 0.367$; **Figure 2B**]. The WT-untreated and 3xTg-AD mice acquired TFC when compared the freezing percentage at intervals 1–3 with baseline performance [$F_{(3,102)} = 173.291$, $p < 0.001$; **Figure 2C**]. The behavioral results demonstrated that the total freezing percentage of all groups after TFC was similar [$F_{(3,34)} = 0.828$, $p = 0.488$; **Figure 2D**], but the retrieval of contextual memory was impaired in the 3xTg-AD mice compared to the untreated WT mice. Injection of 4-PSB-2 significantly enhanced the retrieval of contextual memory of the 3xTg-AD mice compared to that of the untreated 3xTg-AD and sham mice. Moreover, the 3xTg-AD mice treated with 4-PSB-2 showed a significantly higher level of freezing to context than the untreated WT group [$F_{(3,34)} = 20.07$, $p < 0.001$; **Figure 2E**]. Compared to the untreated WT mice, the 3xTg-AD mice did not show significantly impaired memory retrieval of the tone. However, 3xTg-AD mice treated with 4-PSB-2 exhibited significantly increased memory retrieval to tone [$F_{(3,34)} = 2.735, p = 0.059$; **Figure 2F**].

As shown in **Figure 1**, 15 mg/kg 4-PSB-2 significantly increased the apical dendritic spine density in WT mice, we next asked what is the effect of 4-PSB-2 on PSD-95 expression in 3xTg-AD mice. Since PSD-95 is the major scaffolding protein at the excitatory postsynaptic density, a potent regulator of synaptic strength, and is known to be required for retrieval and stability of fear memory (Fitzgerald et al., 2015), we detected PSD-95 as an indirect synaptic marker in total protein samples extracted from the hippocampi of 3xTg-AD mice after TFC. Western blot analysis indicated that the PSD-95 expression level was significantly reduced in the untreated sham 3xTg-AD mice compared with the WT mice [$F_{(3,15)} = 7.601$, $p < 0.01$; **Figure 2G**]. However, it was significantly enhanced after injection of 4-PSB-2 compared with that in the untreated and sham groups.

To further confirm the effect of 4-PSB-2 on synaptic plasticity in 3xTg-AD mice at the cellular level, LTP recording was performed after TFC. The results revealed that fEPSP after LTP

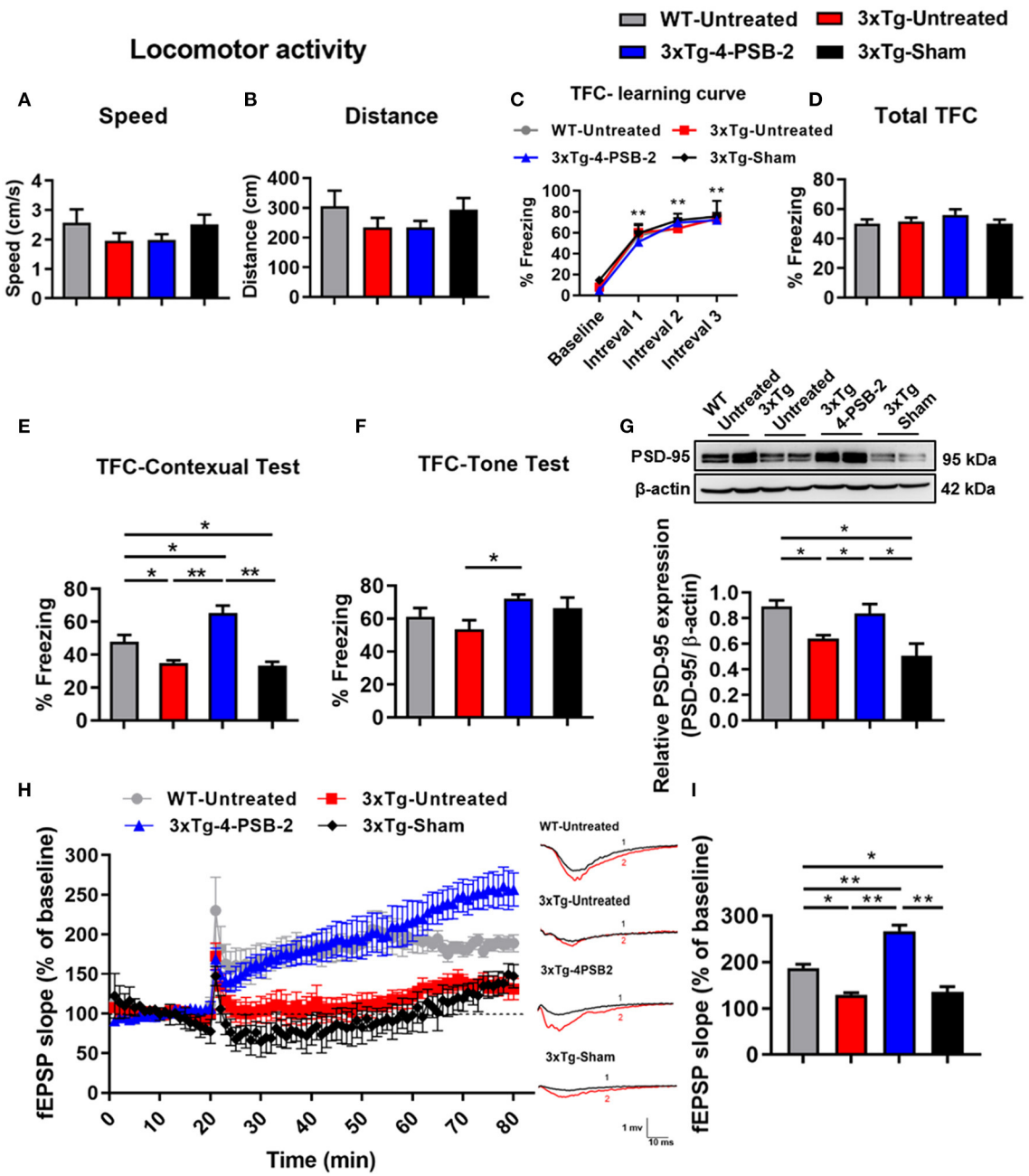


FIGURE 2 | 4-PSB-2 reversed contextual memory retrieval deficit and increased PSD-95 expression and synaptic plasticity in 3xTg-AD mice. **(A,B)** The locomotor activity of WT and 3xTg-AD mice was not significantly different before and after 4-PSB-2 injection. **(C)** The WT and 3xTg-AD mice acquired TFC when compared the freezing percentage at intervals 1–3 with baseline performance. **(D)** The 3xTg-AD mice were subjected to TFC training, and all groups appeared to learn normally. **(E)** The sham 3xTg-AD group exhibited reduced contextual memory retrieval compared to that of the untreated WT group, and the contextual memory retrieval and **(F)** memory retrieval of a tone of the 4-PSB-2-injected 3xTg-AD mice was significantly increased compared to that of the untreated 3xTg-AD mice. **(G)** Western blot analysis showed that PSD-95 expression levels in the hippocampi of the untreated 3xTg-AD and 3xTg-AD sham groups were significantly decreased compared to those in the untreated WT and were significantly increased after the administration of 4-PSB-2. **(H)** LTP was measured in the CA1 region of hippocampal sections of 3xTg-AD mice or WT mice with/without 4-PSB-2 administration for 60 min. **(I)** Last 10 min of the first hour after LTP induction, 4-PSB-2 injection showed restoration of LTP deficit in 3xTg-AD mice and also higher than WT mice. The results are plotted as the means ± SEMs and were statistically analyzed by mixed-design repeated-measures ANOVA followed by Bonferroni test for **(C)**; one-way ANOVA followed by Tukey's test for **(A,B)** and **(D–I)**. *Indicates $p \leq 0.05$, and **Indicates $p \leq 0.001$ between the groups; $n = 8$–11/group for TFC, $n = 4$–5/group for western blot analysis, and $n = 3$–5/group for LTP.

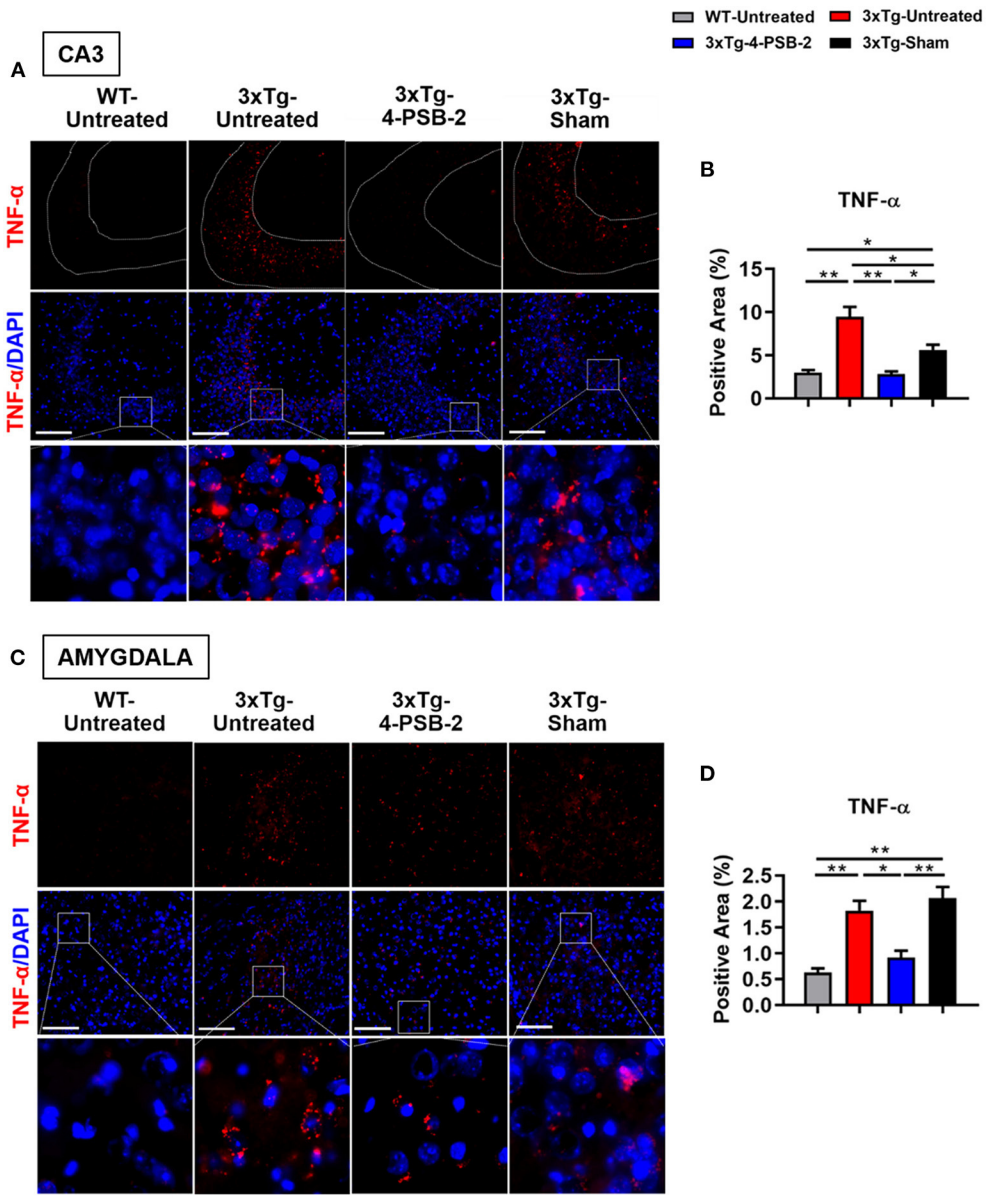


FIGURE 3 | 4-PSB-2 suppressed TNF- α expression in the hippocampal CA3 region and BLA of 3xTg-AD mice after TFC. **(A)** Immunofluorescence staining and **(B)** the quantitative result of TNF-α in the hippocampal CA3 region were significantly increased in the untreated 3xTg-AD and sham groups and were significantly decreased after 4-PSB-2 administration. **(C,D)** The expression of TNF-α in the BLA was significantly increased in the untreated 3xTg-AD and sham groups and was significantly decreased after the administration of 4-PSB-2. The results are plotted as the means ± SEMs. *Indicates $p \leq 0.05$, and **Indicates $p \leq 0.001$ between the groups. TNF-α (red) and DAPI (blue) (nuclei). Bar = 100 μm, and $n = 3$–5/group.

induction in untreated 3xTg-AD and sham mice significantly decreased when compared with the untreated WT group. However, the injection of 4-PSB-2 significantly enhanced fEPSP after LTP induction of the 3xTg-AD mice compared to the untreated 3xTg-AD and sham mice. Furthermore, fEPSP after LTP induction in the 3xTg-AD mice treated with 4-PSB-2 showed a significantly higher than the untreated WT group [$F_{(3,12)} = 40.601$, $p < 0.001$; **Figures 2H,I**].

4-PSB-2 Reduced the Expression Levels of Inflammatory Markers in the Hippocampal CA3 Region and Basolateral Amygdala

Since 4-PSB-2 appeared to be effective in rescuing impaired memory retrieval in 3xTg-AD mice, we next asked whether memory retrieval is linked to expression changes in inflammatory markers in the involved brain region(s). We focused on the hippocampal region and basolateral amygdala (BLA) because

these are the brain areas that play major roles in fear memory formation. The brains of WT and 3xTg-AD mice were collected after TFC and sectioned for immunohistochemical staining. The results revealed that in the hippocampal CA3 region, the expression levels of inflammatory molecules including TNF-α [$F_{(3,55)} = 40.375$, $p < 0.001$; **Figures 3A,B**], COX-2 [$F_{(3,41)} = 9.779$, $p < 0.001$; **Figures 4A,B**], and iNOS [$F_{(3,58)} = 11.68$, $p < 0.001$; **Figures 5A,B**], were significantly increased in the untreated and sham 3xTg-AD group compared with the untreated WT mice. Administration of 15 mg/kg 4-PSB-2 significantly reduced the inflammatory cytokines expression levels. In the BLA, the expression levels of TNF-α [$F_{(3,123)} = 17.713$, $p < 0.001$; **Figures 3C,D**], COX-2 [$F_{(3,47)} = 5.485$, $p < 0.01$; **Figures 4C,D**], and iNOS [$F_{(3,53)} = 8.982$, $p < 0.001$; **Figures 5C,D**] were also increased in the untreated and sham 3xTg-AD groups and were reduced by 4-PSB-2 treatment. The expression levels of COX-2 but not TNF-α and iNOS in sham 3xTg-AD mice were significantly increased in the hippocampal

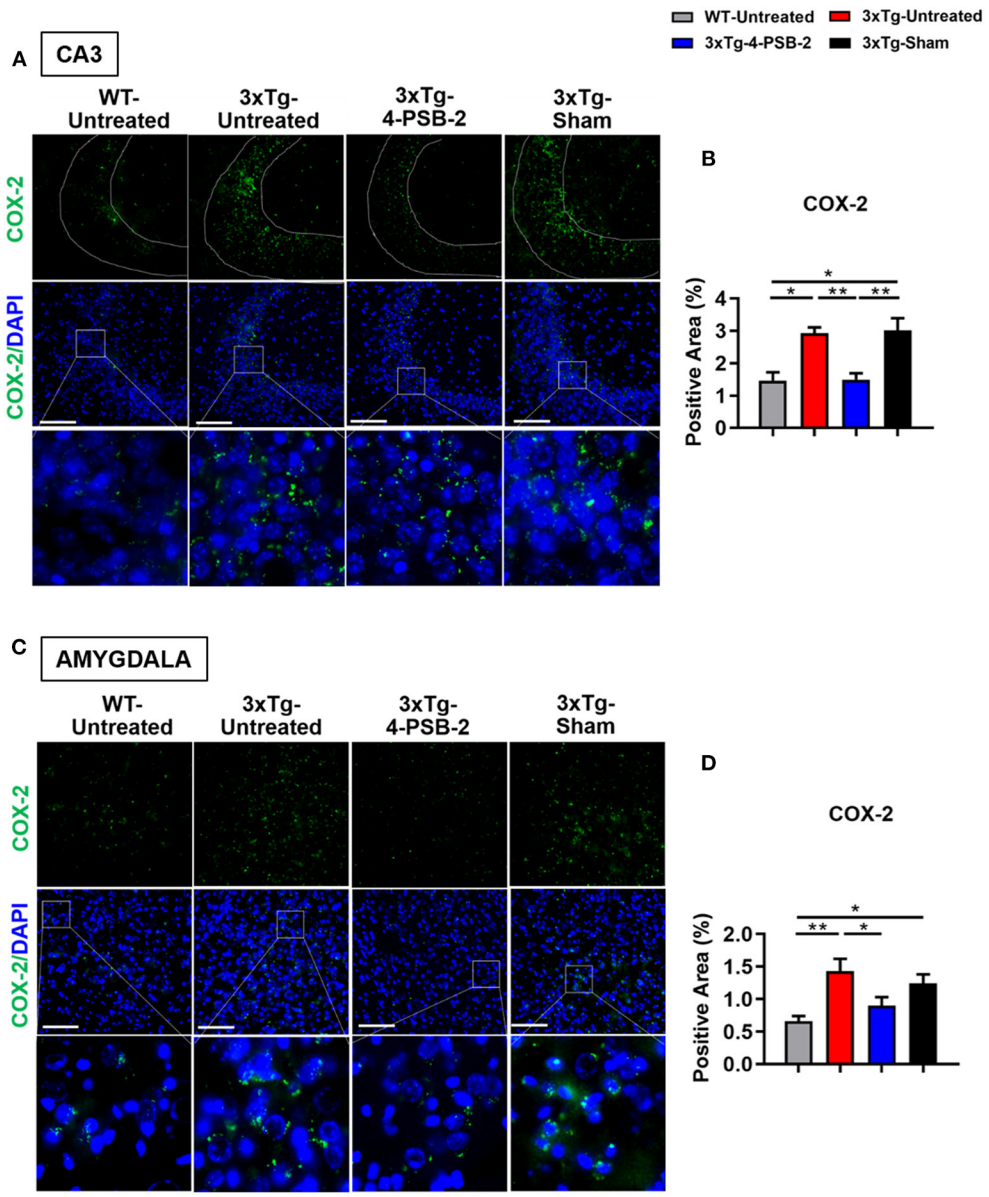


FIGURE 4 | 4-PSB-2 suppressed COX-2 expression in the hippocampal CA3 region and BLA of 3xTg-AD mice after TFC. **(A,B)** The expression of COX-2 in the hippocampal CA3 region was significantly increased in the untreated 3xTg-AD and sham groups and was significantly decreased after 4-PSB-2 administration. **(C,D)** The expression of COX-2 in the BLA of the untreated 3xTg-AD and sham groups were upregulated after TFC. After 4-PSB-2 administration, COX-2 expression was suppressed (compared with that in the untreated 3xTg-AD group). The results are plotted as the means ± SEMs. * indicates $p \leq 0.05$, and **Indicates $p \leq 0.001$ between the groups. COX-2 (green) and DAPI (blue) (nuclei). Bar = 100 μm, and $n = 3$–5/group.

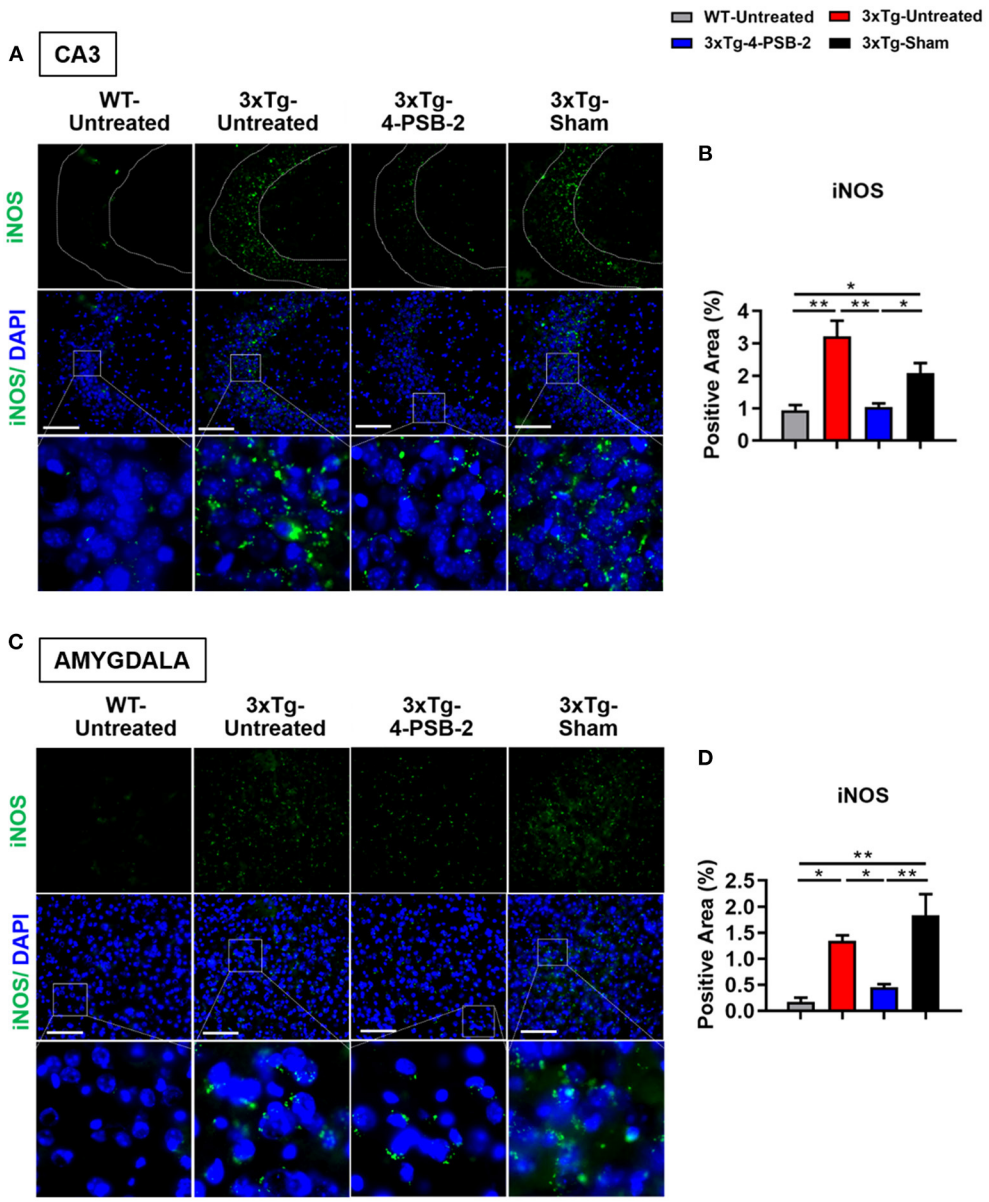


FIGURE 5 | 4-PSB-2 suppressed iNOS expression in the hippocampal CA3 region and BLA of 3xTg-AD mice after TFC. **(A,B)** The expression of iNOS in the hippocampal CA3 region was significantly increased in the untreated 3xTg-AD and sham groups and was significantly decreased after 4-PSB-2 administration. **(C,D)** The expression of iNOS in the BLA of the untreated 3xTg-AD and sham groups were upregulated after TFC. After 4-PSB-2 administration, iNOS expression was also suppressed (compared with that in the sham group). The results are plotted as the means ± SEMs. * indicates $p \leq 0.05$, and **Indicates $p \leq 0.001$ between the groups. iNOS (green) and DAPI (blue) (nuclei). Bar = 100 μm, and $n = 3$–5/group.

CA1 region when compared with the untreated WT mice and were significantly decreased after 4-PSB-2 treatment. In the dentate gyrus (DG), the expression levels of COX-2 and iNOS but not TNF-α in untreated and sham 3xTg-AD mice were significantly increased, but were not decreased after 4-PSB-2 treatment (**Supplementary Figures 2–4**). We also detected the expression of Aβ oligomer (labeled with the A11 antibody) in the hippocampi and the BLA of 3xTg-AD mice. 3xTg-AD mice showed higher Aβ expression than WT mice in the hippocampal CA1 [$F_{(3,87)} = 20.968$, $p < 0.001$; **Figures 6A,B**], CA3 [$F_{(3,71)} = 14.669$, $p < 0.001$; **Figures 6C,D**], dentate gyrus [$F_{(3,73)} = 22.203$, $p < 0.001$; **Figures 6E,F**], and BLA [$F_{(3,62)} = 7.479$, $p < 0.001$; **Figures 6G,H**], but 4-PSB-2 treatment did not change Aβ expression levels.

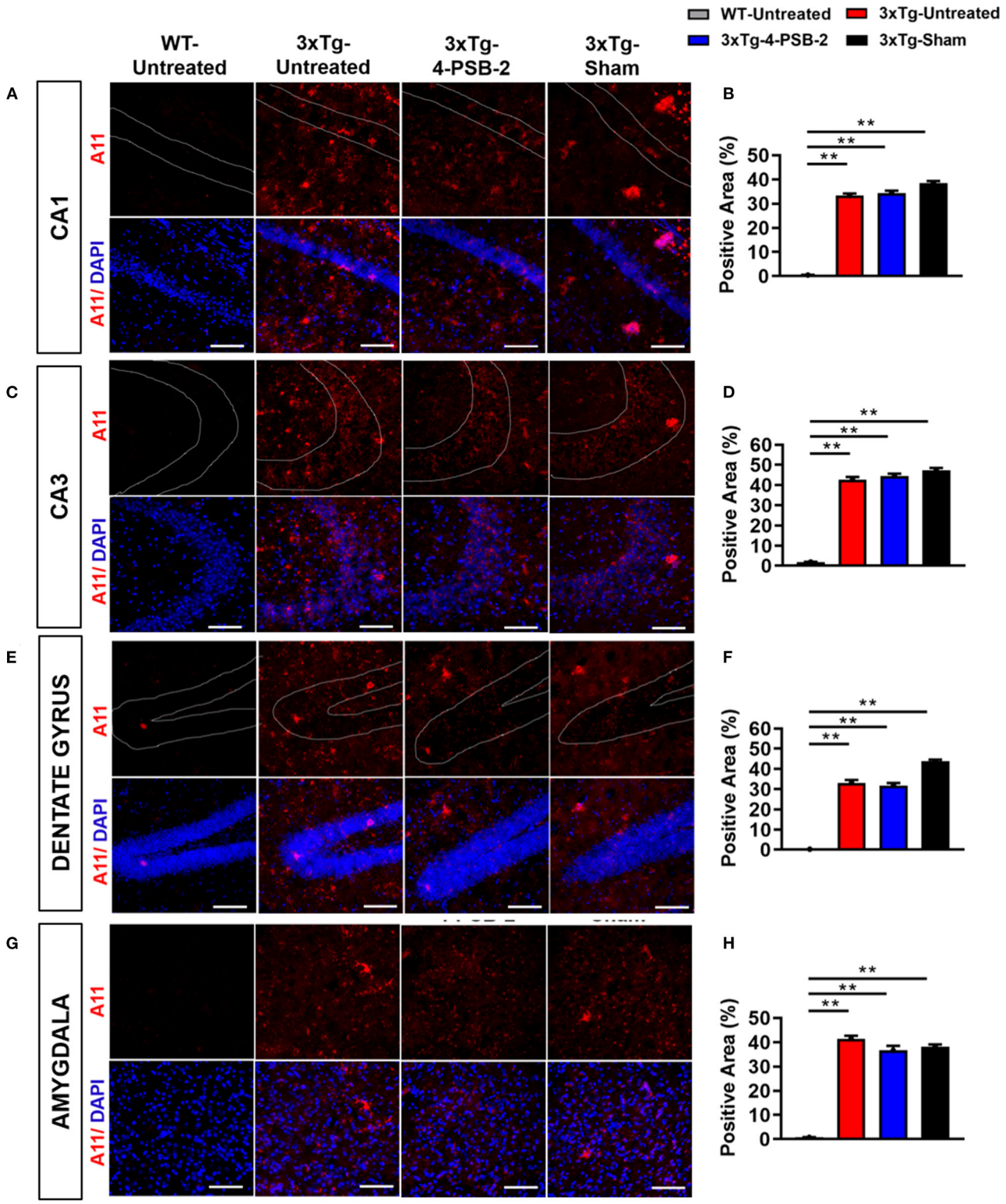


FIGURE 6 | Expression of A11 in the hippocampus and BLA of 3xTg-AD mice after 4-PSB-2 treatment. Immunofluorescence staining of hippocampal **(A,B)** CA1, **(C,D)** CA3, **(E,F)** DG, and **(G,H)** BLA showed that despite the low expression level of A11 in the early AD mice brains, it was increased in all 3xTg-AD mice groups compared to the untreated WT group. After 4-PSB-2 treatment, the expression of A11 in 3xTg-AD mice was not different compared to the untreated 3xTg-AD and sham 3xTg-AD groups. The results are plotted as the means ± SEMs. **Indicates $p \leq 0.001$ between the groups. A11 (red) = Aβ oligomer, and DAPI (blue) = nuclei, Bar = 100 μm.

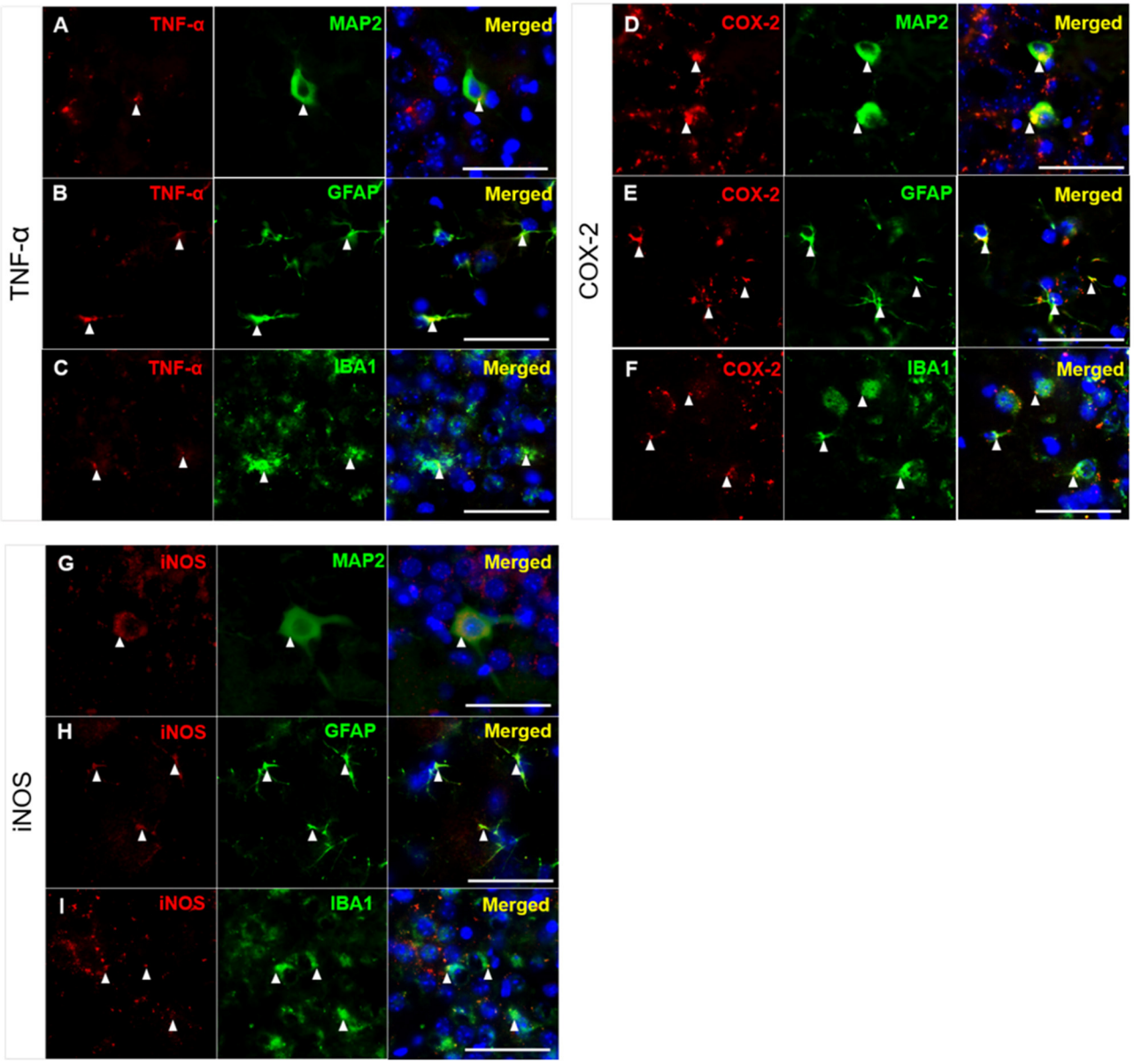


FIGURE 7 | Colocalization of inflammatory molecules with neuronal and glial markers in CA3 region of 3xTg-AD mice. Double immunofluorescence staining for **(A–C)** TNF, **(D–F)** COX-2, and **(G–I)** iNOS with MAP2, GFAP, and IBA1 in the hippocampal CA3 region in 3xTg-AD mice. The results demonstrated that TNF-α, COX-2, and iNOS were detected in the cytoplasm of neurons (labeled with MAP2), astrocytes (labeled with GFAP), and microglial cells (labeled with IBA1), as indicated by the white arrowheads. TNF-α, COX-2 and iNOS (red); MAP2, GFAP, and IBA1 (green); and DAPI (blue) (nuclei). Bar = 50 μm.

Inflammatory Markers Were Expressed in the Cytoplasm of Neurons, Microglia, and Astrocytes in the Hippocampi of 3xTg-AD Mice After TFC

As shown in **Figures 4**, **5**, high levels of TNF-α, COX-2, and iNOS were detected in the hippocampal CA3 region in the untreated 3xTg-AD group after TFC. We next aimed to identify the cell types in which these inflammatory markers were expressed. The brains of 3xTg-AD mice were collected after TFC for double staining with inflammatory markers (TNF-α, COX-2, and iNOS), a neuronal marker (MAP2) or glial markers (GFAP for astrocytes and IBA1 for microglia). TNF-α was colocalized with MAP2 (**Figure 7A**), GFAP (**Figure 7B**), and IBA1 (**Figure 7C**) in the cytoplasm. TNF-α downstream targets, COX-2 and iNOS (Song et al., 2012), were also found to be colocalized with MAP2 (**Figures 7D,G**), GFAP (**Figures 7E,H**), and IBA1 (**Figures 7F,I**) in the cytoplasm. The results indicate that these inflammatory markers were expressed in all three cell types.

DISCUSSION

In the present study, we report that 4-PSB-2 significantly rescues the impairment of fear memory retrieval in 3xTg-AD mice and increases dendritic spine density and LTP through the suppression of several inflammation markers in the BLA and hippocampus (**Graphical Abstract**), particularly in the CA3-region. Studies on AD patients have confirmed that atrophy (Basso et al., 2006) and functional disconnectivity (Ortner et al., 2016) of the hippocampus and BLA are associated with memory decline (Basso et al., 2006; Ortner et al., 2016). These two brain regions are known to be important for the retrieval and mediation of fear memory in non-AD mice (Anagnostaras et al., 2001; Gale et al., 2004; Lu et al., 2009). More internal synaptic connections existing in the hippocampal CA3-region than in other regions has been reported (Cherubini and Miles, 2015). Therefore, the loss of neurons and synaptic connectivity within the CA3-region may reduce the precision of memory retrieval (Chadwick et al., 2014) in AD.

We further found that the inflammatory markers in the CA3-region were expressed in neurons, microglia, and astrocytes of 3xTg-AD mice. These inflammatory molecules seem to mediate cross-talk among these three types of cells. Microglia are glial cells that are responsible for homeostasis and immune defenses in the brain and can become polarized toward the proinflammatory M1 and immunosuppressive M2 phenotypes by Aβ deposition (Fakhoury, 2018). In AD, an excess of Aβ deposition disrupts the balance between M1 and M2 microglia, resulting in the overexpression of proinflammatory molecules (IL-1β, TNF-α, iNOS, and IL-6), in turn causing neuronal damage (Tang and Le, 2016). The increase of proinflammatory molecules such as LPS, IL-1β, TNF-α, and IFN-γ also interfered with the microglia's capacity to remove Aβ by suppressing microglial endocytic (Sole-Domenech et al., 2016) and phagocytic activities (Koenigsknecht-Talboo and Landreth, 2005). The microglial endocytosis can be improved by applying 40 Hz gamma oscillations to the brains of AD mice (Iaccarino et al., 2016). The microglial phagocytic activity can be improved with anti-inflammatory treatment (Koenigsknecht-Talboo and Landreth, 2005). Astrocytes are also activated by Aβ and then secrete excessive inflammatory cytokines leading to neuronal injury (Fakhoury, 2018). Several studies have revealed that inflammatory cytokines such as the TNF-α (Yu et al., 2017), COX-2 (Chen et al., 2013), and iNOS (Lisboa et al., 2015) played important roles in regulating contextual fear memory. It is possible that in AD or aging mice, the inflammatory response is enhanced and lasts for long, which leads to over-activation of microglia and astrocytes in the CA3-region, resulting in neuronal function deficit, synaptic loss, synaptic transmission inefficiency (Mariani et al., 2017), and learning and memory impairment (Lana et al., 2016). Moreover, the brain areas other than the CA3-region and BLA are also affected by an enhanced inflammatory response in this transgenic AD mouse strain upon aging (Backman et al., 1999; Kinney et al., 2018). Therefore, more age-dependent memory tests need to be performed in AD mice to elucidate the relationship between changes of the inflammatory response in different brain regions.

The present study shows that 4-PSB-2 acts as a memory enhancer to increase contextual fear memory, elevate dendritic spine density, PSD-95 expression, and LTP in the hippocampus. The PSD-95 is a key protein that increases synaptic strength and the efficiency of the formation and retrieval of fear memory (Hering and Sheng, 2001; Fitzgerald et al., 2015; Huang et al., 2018). It is also known to regulate glutamatergic plasticity at the postsynaptic site (El-Husseini et al., 2000) and stabilizes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) to promote synaptic function and spine growth (Chen et al., 2011). The reduction of PSD-95 and synaptic spine density can interfere with hippocampal LTP in AD mouse models (Tu et al., 2014; Gu et al., 2016). LTP is a well-accepted cellular representation of the synaptic plasticity indicating the efficiency of learning and memory. In the present study, we recorded reduced LTP from the Schaffer-collateral pathway, which sends efferent fiber from the CA3 to the CA1 regions of the hippocampus (Kumar, 2011; Cherubini and Miles, 2015). This phenomenon is in line with previous findings from other AD mouse models such as Tg2576 mice (Comery et al., 2005), APP/PS1 mice (Gu et al., 2016), and 5XFAD mice (Kimura and Ohno, 2009). Interestingly, the impaired LTP was reversed by 4-PSB-2 administration that has anti-inflammatory effects, indicating a relationship between levels of inflammation response with synaptic transmission.

The 4-PSB-2 has a similar chemical structure as BAY 11-7082 reported in a previous study (Lee et al., 2012), which can also suppress the expression of inflammatory proteins, including IκB, NF-κB, and Akt. The structure of BAY 11-7082 varies slightly from that of 4-PSB-2, as it contains a reactive Michael acceptor and a cyanide group in place of a ketone group. The structural similarities of the compounds probably underlie their similar functions in suppressing the inflammatory response. The 4-PSB-2 is likely able to pass through the blood-brain barrier since it is lipophilic and small; its molecular weight (180) is <400 Daltons (Pardridge, 2012). Our results are consistent with previous studies (Moy et al., 2004; Nakatani et al., 2009; Pearson et al., 2010), in which the "social recognition" of C57BL/6 remained intact with alterations to the "social novelty" behavior. The C57BL/6 mice in our study showed an increasing trend in "social novelty" behavior though not significant. This behavior was improved after 4-PSB-2 treatment. The 4-PSB-2 was administered immediately after TFC because it is important for memory consolidation and inflammatory response induction (Barrientos et al., 2002; Johansen et al., 2011; White et al., 2020). Efficient memory consolidation in the hippocampus is known to help better memory retrieval (Bosshardt et al., 2005). Post-injection behavioral evaluation did not indicate any effect that 4-PBS-2 seems to cause on anxiety test and locomotor activity of WT and 3xTg-AD mice, which suggests that 4-PSB-2 may be a good drug lead. It is noted that a single dose of 4-PSB-2 used in this study did not significantly reduce Aβ oligomer expression in the hippocampus (Shin et al., 2014) and BLA of 3xTg-AD mice; therefore, further time course studies of pharmacokinetics, toxicity, metabolism, and the effects of multiple doses are necessary.

In conclusion, the excessive inflammatory response may be associated with impairment of fear memory retrieval occurred in early-stage AD. Our results demonstrate that targeting and reducing the inflammatory response in the brain with the 4-PSB-2 may be a promising approach to prevent and treat related memory symptoms of AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Tzu Chi University.

AUTHOR CONTRIBUTIONS

IL and PV developed hypotheses, designed and performed experiments, analyzed data, prepared **Figures 2**–**7**, **Graphical Abstract**, and wrote manuscript. KG developed hypotheses, performed the experiment, analyzed data, and prepared **Figure 1**. S-PH and SC developed hypotheses, designed experiments, and helped technical support. CE performed the experiment, analyzed data, and prepared **Figure 1A** and **Table 1**. PS helps with experimental designs, performed the experiment, and prepared the manuscript. Z-HW provided the compound and helped interpret results. All authors read and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.615079/full#supplementary-material

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Neuroimaging, Behavioral, and Gait Correlates of Fall Profile in Older Adults

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Prior research has suggested that measurements of brain functioning and performance on *dual tasks* (tasks which require simultaneous performance) are promising candidate predictors of fall risk among older adults. However, no prior study has investigated whether brain function measurements during dual task performance could improve prediction of fall risks and whether the type of subtasks used in the dual task paradigm affects the strength of the association between fall characteristics and dual task performance. In this study, 31 cognitively normal, community-dwelling older adults provided a self-reported fall profile (number of falls and fear of falling), completed a gait dual task (spell a word backward while walking on a GaitRite mat), and completed a supine dual task (rhythmic finger tapping with one hand while completing the AX continuous performance task (AX-CPT) with the other hand) during functional magnetic resonance imaging (fMRI). Gait performance, AX-CPT reaction time and accuracy, finger tapping cadence, and brain functioning in finger-tapping-related and AX-CPT-related brain regions all showed declines in the dual task condition compared to the single task condition. Dual-task gait, AX-CPT and finger tapping performance, and brain functioning were all independent predictors of fall profile. No particular measurement domain stood out as being the most strongly associated measure with fall variables. Fall characteristics are determined by multiple factors; brain functioning, motor task, and cognitive task performance in challenging dual-task conditions all contribute to the risk of falling.

Keywords: falls, gait, cognitive, fMRI, motor function

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INTRODUCTION

Falls affect more than 30% of older adults. Therefore, it is not surprising that falls in this population are the leading cause of non-fatal injury in this population (Centers for Disease Control and Prevention, 2014), while also a major cause of fatal injury. Falls are also associated with declines in functional status and social activity (Stel et al., 2004), as well as significant financial burdens due to consequent health care utilization (Alexander et al., 1992; Hoffman et al., 2017). Unfortunately, identifying older adults at increased risk of falling is especially challenging. A large set of studies have assessed fall risk using a variety of different predictors. Studies have assessed fall risk based

on gait and slip responses (Rahul and Thurmon, 2017), gait and fall history (Toulotte et al., 2006; Ansai et al., 2017), musculoskeletal function tests (MacRae et al., 1992), and cognitive performance (Hausdorff et al., 2001; Verghese et al., 2002; Springer et al., 2006; Holtzer et al., 2007; Herman et al., 2010; Buracchio et al., 2011). Despite some studies showing some promise as to its usefulness to predict falls using these measurements, it still has not been proven to be flawless; hence the vast number of studies using different metrics to assess fall risk. Most studies are able to discriminate with modest accuracy fallers from non-fallers. For example, one posturography-based approach discriminated multiple-fallers from non-multiple-fallers with 85% accuracy (Howcroft et al., 2017). Improving accuracy of fall risk prediction systems is especially important, because interventions targeted to high-fall-risk individuals have the potential to reduce fall risk through strength and balance training, built environment modification, and other methods [for a complete list, see Stevens and Burns (2015)].

One key limitation of measurements that focus on a single domain, such as cognition, motor function, or sensation, is that they do not simulate realistic scenarios in which complex motor tasks must be completed at the same time as a distracting and unrelated cognitive task. Distraction scenarios are common among older adults–consider walking while answering a cell phone–and frequently lead to falls. Laboratory-based dual task paradigms combining gait with a cognitive load have been devised to simulate such scenarios (Hausdorff et al., 2001; Springer et al., 2006; Allali et al., 2007; Yogev-Seligmann et al., 2008; Herman et al., 2010; MacAulay et al., 2014, 2015; Ansai et al., 2017; Rahul and Thurmon, 2017). Other dual-task paradigms have focused on concurrent execution of two, unrelated motor tasks (Toulotte et al., 2006). While prior studies have identified associations between these dual-task measures and falls, it is unclear how these measures compare to competing measures, such as brain functional measures, in terms of strength of association with falls.

Functional neuroimaging during performance of dual tasks provides an alternative means of assessing fall risk by identifying deficiencies in the functioning of brain networks that contribute to dual task performance, even in the absence of overt deficiencies in task performance. Brain function deficiency measures are promising as fall predictors because these inadequacies could culminate in cognitive and motor deficiencies in more varied and complex real-world environments occurring outside the laboratory. Furthermore, when brain function deficiencies are identified, often, without intervention, they become progressively worse over time. Although previous research has assessed the brain functional correlates of dual-task performance, including the rhythmic motor components of distracted gait (Holtzer et al., 2011; Van Impe et al., 2011; Johnson and Shinohara, 2012; Doi et al., 2013; Leone et al., 2017; Papegaaij et al., 2017), and brain activity during walking and reciting letters (Verghese et al., 2017) to our knowledge none of these studies determined whether the brain functional measures predicted fall risk based on retrospective fall history.

The goal of this study was to take a step toward improved fall prediction by assessing performance on a gait dual task, brain functioning during a stationary dual task, and performance on the stationary dual task as an independent correlate with fall history. We collected the stationary task data from 31 cognitively normal older adults after they had already completed a minimum of three yearly clinical evaluations, each of which included a self-reported fall profile and gait during dual task performance. To our knowledge, this is the first study that simultaneously assessed distracted gait measures as well as performance and brain functional measures on a stationary dual task, to identify correlates with fall history.

MATERIALS AND METHODS

Participants

All participants were enrolled in the Louisiana Aging Brain Study (LABrainS), a longitudinal observational cohort maintained by the Institute for Dementia Research and Prevention (IDRP) at the Pennington Biomedical Research Center (PBRC) designed to investigate cognitive, motor, and affective changes among cognitively normal older adults aged 60–85. Participants throughout Louisiana were recruited into LABrainS through typical media sources such as newspaper advertisements and television, in addition to outreach done by the IDRP. Exclusion criteria from LABrainS included: a history of neurological or untreated health conditions (e.g., Parkinson's disease and/or a traumatic brain injury) that might cause cognitive impairment, or a Geriatric Depression Scale ≥ 6 [15 item version, (Yesavage et al., 1982]. A total of 416 LaBrains participants had at least 3 years of complete gait, neuropsychological, and fall data at the time this study started. A set of 50 of these individuals were recruited into the current study. Additional exclusion criteria for this study were contraindications to MRI, left handedness, and vision not corrected to 20/20. Of the 50 participants, six did not provide analyzable MRI data due to excessive head motion or similar acquisition issues. An additional 13 participants did not follow task directions and therefore did not provide analyzable data. Therefore, data was processed and analyzed from the remaining 31 participants; see **Supplementary Figure 1**. Informed consent was obtained from participants prior to their visit assessments. This study was approved by the PBRC Institutional Review Board. Characteristics of the 31 participants are shown in **Table 1**. Average age for the participants was 73.0 $\pm$ 6.7 years, with 25 females and six males. Cognitive and motor status was assessed using the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (Randolph et al., 1998) and the Short Physical Performance Battery (SPPB) (Guralnik et al., 1994), respectively.

Fall History

Fall history among LaBrainS participants was collected at each yearly clinical visit. Since the term "fall" may have different meanings to different individuals, the definition used in this study was consistent with established recommendations: "times in which an individual unexpectedly lost balance and unintentionally came to rest upon the ground, floor, or other object" (Lamb et al., 2005). This did not include those times that

TABLE 1 | LABrainS participants characteristics and demographics.

Age	73.0 ± 6.7 years
Sex	25 female, 6 male
Ethnicity	30 white, 1 black/African American
RBANS	111.9 ± 17.6
SPPB	11.14 ± 1.60
Falls yes/no	14 yes, 17 no
Number of falls ever	1.48 ± 2.56
Fear of falling	0.19 ± 0.37
Time between successive gait measurements	12.39 ± 1.86 months
Time between most recent gait measurement and fMRI acquisition	2.19 ± 4.27 months

participants were able to regain their balance before coming into contact with the ground. Each participant completed a survey of fall history at each yearly visit. Collected data items identified fear of falling (as a binary), times fallen in the past 12 months, times fallen in the past 2 years, total times fallen over the lifespan, and categorization of the person as a "faller" or a "non-faller" (i.e., a person who did or did not report at least one fall over the lifespan). This retrospective fall data was included in analysis.

Gait Dual Task Acquisition and Analysis

Gait data was acquired at each clinical visit. The GAITRite system (CIR Systems, Inc., Sparta, NJ, United States), an electric sensor walkway, was used for collection of gait data. In the single task condition, participants were instructed to walk across the walkway "using their normal everyday walking speed." In the dual task condition, participants were additionally instructed to spell a word backward aloud while walking (for list of words, see the GAITRite manual). Stride length (the line of progression between two consecutive footprints of the same foot) and step time (time elapsed between the contact of one foot on the floor to the opposite foot's contact) served as gait performance measures in single and dual task conditions. Average stride length and step time within the single and dual task conditions were calculated at each visit. In our analysis, we used these specific gait variables: average stride length for single task, dual task, and dual-task hit, average step time for single task, dual task, and dual-task hit. GAITRite data has been analyzed successfully in older adult cohorts (MacAulay et al., 2014; Brown et al., 2015).

Stationary Dual Task Design

Participants performed two tasks with button boxes, separately and then simultaneously, while lying supine in an MRI machine. Button boxes were held in the left and right hands over the chest area with the middle and index fingers of each hand being used to perform the tasks. The first task was self-paced finger tapping for 90 s. On the instruction screens, participants were told to tap in cadence with a flashing box that appeared for 0.03 s every 0.4 s, alternating the tapping between left middle and index fingers, for a total of 10 s, before being told to tap at a natural cadence without a visual cue. The second task was the AX-continuous performance task (AX-CPT), a streaming letter memorization and recognition task (Servan-Schreiber et al., 1996). In each trial of this task, participants viewed a pair of letters serially on the screen; a white letter ("cue") followed by a blue letter ("probe"), both appearing on a black background. The instruction was to press the right index finger button when the probe letter was X immediately following an A cue. The left index finger button should be pressed for any other combination of cue and probe letters. There were four types of trials, depending on the cue and probe letter: AX trials (A cue, X probe), BX trials (non-A cue, X probe), AY trials (A cue, non-X probe), and BY trials (non-A cue, non-X probe). Non-A cues were drawn from this set of letters: E, P, G, R, S, and V. Non-X probes were drawn from this set of letters: F, J, M, Q, and U. The cue was shown for 0.5 s, then disappeared to a black screen for a 5.5 s response period, then the probe was shown for 0.5 s, followed by a 5.5 s response period. Participants completed 36 AX-CPT trials in the single task condition. Next, the finger tapping and AX-CPT tasks were performed simultaneously, consisting of 36 AX-CPT trials with the right hand and self-paced finger tapping with the left hand. Behavioral data was acquired with MATLAB 2016a with Psychophysics Toolbox and the Fiber Optic Response Pad (FORP) button box system on non-auto release button mode. A timestamp was generated every 10 ms indicating if a button was currently depressed, and this data was analyzed using in-house software. Only the first button press was recorded as a response.

Stationary Dual Task Behavior Analysis

For the finger tapping task, the average number of taps per second (tapping cadence), its standard deviation, and the time the button was held down were calculated. For the AX-CPT task, we calculated the overall accuracy as the number of cues and probes answered correctly divided by the total number of cues and probes. This was further categorized into accuracy by trial type. Reaction time was the time difference between the onset of the cue or probe and when the button was pressed. The dual-task hit was the percentage change going from single task to dual task, and this was calculated for accuracy and reaction time for each trial type, as well as overall. For finger tapping, we calculated tap cadence as the mean number of seconds in between taps. We calculated tap cadence within a sliding window of 15 s duration, and calculated tap consistency as the standard deviation of tap cadence across all sliding window locations. We also calculated tap duration as the mean amount of time (in seconds) that the button was held down during a tap. Each of these measures was calculated separately in single and dual task conditions. The dual-task hit was also calculated for each of the tapping measures.

fMRI Acquisition

Functional magnetic resonance imaging (fMRI) scans were acquired on a 3T MRI scanner (General Electric, 750 W Discovery, 32-channel quadrature head coil) using a blood oxygen level dependent echo-planar imaging (BOLD-EPI) pulse sequence. Participants wore both a pulse oxygenation sensor and respiratory monitoring belt during scanning

to correct for cardiac and respiratory influences on fMRI signals. Key acquisition parameters were the following: voxel size: 3.4 mm × 3.4 mm × 3.5 mm, TR: 3 s, number of slices: 52, and TE: 30 ms. Structural images required for functional data analysis were obtained using a T1-weighted magnetization-prepared gradient echo pulse sequence with the following parameters: TR: 8.7 ms, TE: 3.8 ms, FA: eight degrees, number of slices: 176, and voxel size: 1 mm × 1 mm × 1 mm.

fMRI Data Analysis

Functional MRI data was analyzed with MATLAB R2016a and the Statistical Parametric Mapping 12 (SPM12) toolbox. Preprocessing steps included these steps: realignment for head motion correction, co-registration to the structural scan, slice timing corrections, smoothing using a 6 mm full width at half maximum Gaussian kernel, and warping to the Montreal Neurological Institute (MNI) template. We used the RETROICOR algorithm to remove cardiac and respiratory components of each time series. Time points representing volumes with excessive head motion (defined as greater than 1.5 mm of translation or 1.5 degrees of rotation from the previous time point) and activation spike artifacts (defined as global mean brain activation greater than 2.3 standard deviations above the mean across all time points) were removed from analysis. The data for each participant was entered into a first-level voxel-wise analysis using the general linear model. Each trial was modeled as a boxcar function convolved with the canonical hemodynamic response function that began at the onset of the stimulus presentation. First level beta maps, performed at the single participant level, quantified differences in BOLD signal between different components of the single and dual task. Region-of-interest (ROI) analysis was performed using ROIs obtained from other studies (Witt et al., 2008; Lesh et al., 2013; Lopez-Garcia et al., 2016), see **Supplementary Table 1**. For each ROI, the set of contrast values within a 5 mm radius sphere surrounding the ROI location was averaged to provide the ROI summary. These ROI-level summary measures were the primary fMRI measures of interest in the statistical analysis.

Statistical Analysis: Analysis of Dual-Task Hits

For each of the behavioral, fMRI, and gait summary variables, we calculated the mean and standard deviation across participants in single-task and dual-task conditions. We quantified the "dual-task hit" for each measure in terms of the signed percent difference in the measure between single-task and dual-task conditions. We used one-sample *T* tests to assess whether the means of such dual-tasks hits differed significantly from zero. Among behavioral measures, we then assessed correlations between corresponding reaction time and accuracy dual task hits to explore whether participants tended to exhibit decrements in speed, accuracy, or both. In addition, we assessed correlations between AX-CPT dual task hits and finger tap dual task hits to explore whether participants tended to experience performance decrements in one of the tasks or both simultaneously.

Statistical Analysis: Predictors of Fall Profile

We took an incremental model building approach to assessing gait, behavioral, and imaging predictors of fall profile (Carmichael et al., 2012). Specifically, for each of the predictor variables, separate linear regression models were estimated with that variable as the sole predictor and one of the fall variables (presence or absence of fall history, number of falls over the past year, fear of falling, and total number of falls) as the outcome. Independent predictor variables entered into the single predictor models included the single-task, dual-task, and dual-task hit of accuracy, reaction time, tap cadence, tap duration, and tap consistency. *P* values less than 0.05 in those models were viewed as statistically significant. For fall outcomes with statistically significant regression models from more than one measurement domain (gait, behavior, imaging), a combined model was estimated including the most significant (i.e., lowest *P* value) predictor among the significant predictors in that domain. The combined models sought to assess whether any specific measurement domain showed a pattern of relatively stronger association with the fall variables, compared to other domains. Power was determined by using a correlation power analysis.

RESULTS

Stationary Dual Task Performance

AX-continuous performance task accuracy decreased significantly in the dual task condition, compared to the single task condition, both overall and in every trial type except BY (see **Table 2** and **Supplementary Table 2**). In addition, AX-CPT reaction time increased significantly in the dual-task condition compared to the single-task condition (see **Table 2**), but this effect was mainly driven by increased reaction time on the BY trials. Tap cadence was slower, tap consistency was greater, and tap duration was longer in the dual-task condition compared to the single-task condition. Although the AX-CPT accuracy dual-task hit was substantial on average, there was marked inter-individual variability, with some participants maintaining high levels of accuracy (**Figure 1**). There was limited evidence of a significant relationship between dual-task hits to AX-CPT performance and dual-task hits to finger tap performance (see **Supplementary Table 3**).

Stationary Dual Task fMRI Data

Eighteen of the pre-defined ROIs showed significantly reduced fMRI activation in the dual-task condition compared to the AX-CPT single-task condition. Of these ROIs, six were AX-CPT ROIs and 12 were finger tapping ROIs (**Figure 2**). No ROIs showed significantly greater fMRI activation in the dual-task condition compared to the AX-CPT single-task condition. When the fMRI data was partitioned into AX trials vs. non-AX trials, a similar pattern of reduced ROI activation emerged within each partition separately (see **Supplementary Figure 2**).

TABLE 2 | AX-CPT accuracy, AX-CPT reaction time, and finger-tapping performance in the single and dual task conditions, along with their standard errors.

	Single	St. error	Dual	St. error	% diff	*p*-value
AX-CPT accuracy	94.6	1.4	82.6	3.6	−12.7	<0.001**
AX-CPT reaction time (s)	0.74	0.04	0.80	0.04	9.0	0.04*
Tap cadence (s/tap)	0.48	0.02	0.54	0.03	12.6	0.001**
Tap consistency (s/tap)	0.05	0.01	0.25	0.04	429.1	<0.001**
Tap duration (s)	0.23	0.02	0.29	0.02	25.4	0.001**

*Percent difference in the mean value and p-values for one-sample T tests are shown in the right column. * = significant at the p = 0.05 level, ** = significant at the p = 0.001 level.*

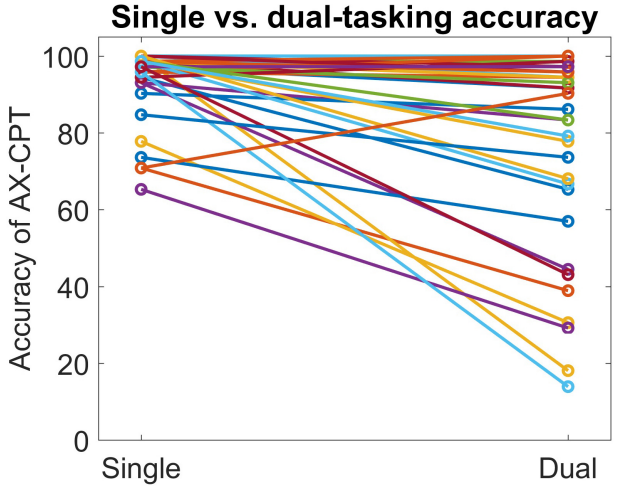

FIGURE 1 | Scatterplot depicting inter-individual variability in dual task hits to AX-CPT accuracy.

Gait Dual Task Data

Gait step length decreased in the dual task (walking plus cognitive task) compared to the single task by 7.3% ($p < 0.001$). Gait step time increased significantly by 6.2% ($p < 0.001$). In addition, the standard deviations of both the step length and step time increased during the dual task compared to the single task, by 4.5 and 8.0%, respectively.

Individual Predictors of Fall Profile

Multiple predictors, spanning multiple measurement domains, were significantly associated with each of the four fall profile variables in single-predictor regression models (see **Table 3**). Step lengths in the single- and dual-task conditions were significant predictors from the gait domain, while dual-task hits to finger tap cadence and an AX-CPT reaction time variable were significant predictors from the behavioral domain. 12 imaging ROIs (four AX-CPT ROIs and eight motor cortex ROIs) had dual-task fMRI hits that were significant predictors of total number of falls, while two imaging ROIs (one AX-CPT and one motor cortex ROI) had dual task hits that were significant predictors of faller/non-faller status. Specifically, the right superior parietal cortex ROI (of the AX-CPT set) had a dual task hit that correlated with three of our fall variables. No gait dual-task hit variables were correlated with falls.

Simultaneous Predictors of Fall Profile Variables

In multiple predictor models containing the most-significant gait, behavioral, and imaging predictors, there was no clear pattern suggesting that predictors from one domain or another provided the strongest associations with fall profile variables. In the multiple-predictor model with fear of falling as the outcome measure, both a stationary dual-task performance measure (tap cadence dual task hit) and an fMRI variable (L SMA dual task hit) were significant predictors with nearly equivalent p values ($p = 0.004$ for each), which shows increased power from the single-predictor models. In the multiple-predictor model with falls in the past year as the outcome measure, only one stationary dual-task performance measure (tap cadence dual task hit) was a significant predictor ($p = 0.02$). In the multiple-predictor model with total number of falls as the outcome measure, both of the gait dual task or fMRI predictor variables were significant predictors. In the multiple-predictor model with faller/non-faller status as the outcome variable, only one stationary dual task performance variable (BY reaction time dual task hit) was a significant predictor from the imaging domain, and the right dorsal premotor cortex three ROI was significant from the imaging domain. The power of these multiple predictor models was over 80% in all cases except for faller/non-faller status as a binary, which yielded 75% power.

DISCUSSION

The key finding of this study is that multiple domains of measurements including gait, neuroimaging, and dual cognitive/motor tasking all provided significant and independent information that explained variability in fall profile variables, including fear of falling and history of falls. The key implication of this finding, if extended to prospective cohorts, is that even if highly sophisticated measurements such as functional MRI of the brain are available, there is still value in a fall risk assessment that is as multi-factorial as possible. The finding emphasizes that falling is influenced by a wide array of factors, including central control of cognitive and motor resources, skeletal muscle function, peripheral nervous system activity, and other contributors not addressed here such as the built environment.

The hypothesis driving our dual-task approach is that in some older adults, performing an additional cognitive task at the same time as standing or walking can reduce the ability

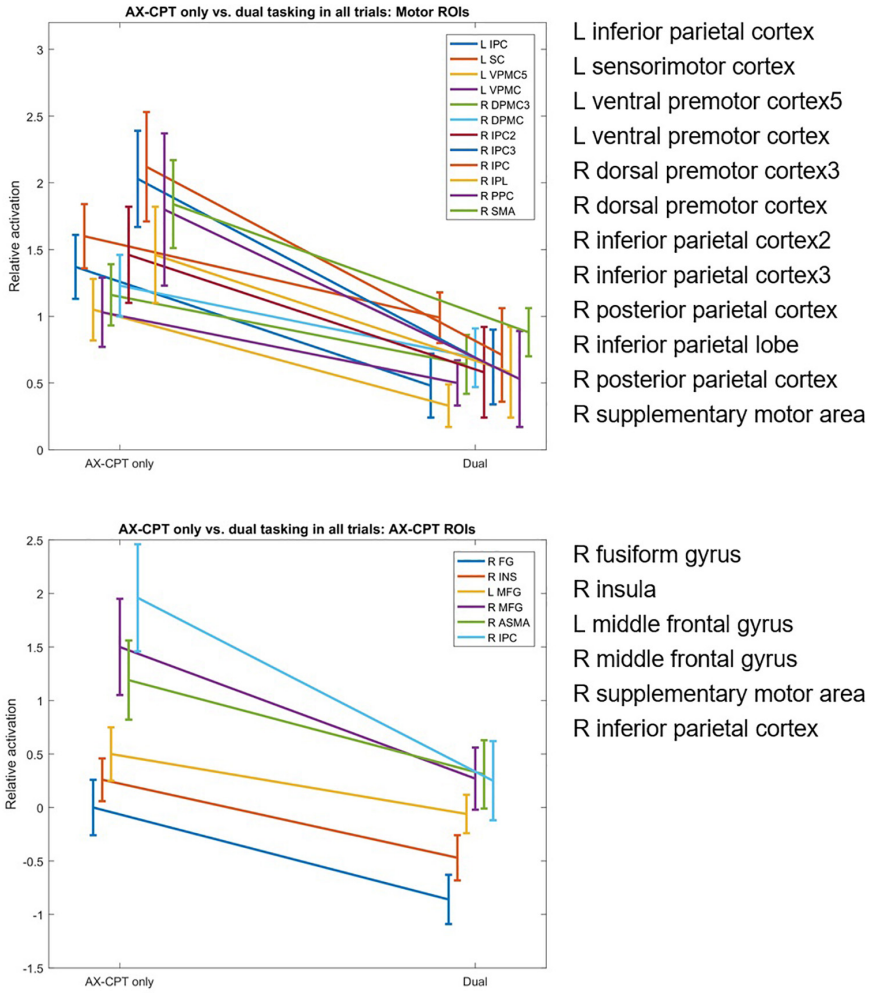

FIGURE 2 | Dual-task hits in fMRI activation in AX-CPT-related and finger-tap-related regions of interest, for single AX-CPT task vs. dual AX-CPT/tapping task.

to control balance and limb movements to such an extent that the risk of falling is increased. In this way, we were following the lead of a large existing body of literature about dual task performance which posited that interference between disparate task-related cognitive processes (Pashler, 1994), or processing "bottlenecks" (cognitive resources that at any moment can be utilized by one task or the other but not both) (Pashler, 1984) led to decrements in performance on either of the simultaneous tasks. Although we possessed diverse indicators of dual-task decrements in task performance (from task responses, gait performance, and brain functioning), no single type of indicator was predominant in providing the information about fall profile. One possible reason is that the different domains of dual-task performance represent different aspects of dual-task performance, each of which is relevant to falls. Specifically, the cognitive component of the stationary dual task addressed the ability to perform a continuous reactive task that requires constant monitoring, while the cognitive component of the distracted gait task addressed spontaneous language generation. Both types of cognitive performance are relevant to falls and have distinct brain circuitry underpinning them. The imaging variables, meanwhile, address the ability to recruit brain resources from established task-related circuitry to handle the increased demands of the dual task, which could independently contribute to the ability to react to shifting cognitive demands during avoidance of falls. Future work should explore whether there are additional aspects of dual-task performance that could provide even more independent information about fall profile.

The current findings are aligned with, and extend, prior findings on predictors of fall profile variables. The associations between gait variables and fall profile replicate previously published findings in a larger group of individuals from the same LaBrainS cohort (MacAulay et al., 2015), which found that gait step length decreased significantly in the dual task condition compared to the single task condition, and that dual task step length significantly differed between those self-reporting a history of falls vs. those who did not. Dual-task test performance has been shown to be associated with fall risk in many studies with different gait performance measures such as gait variability and gait velocity [for a systematic review, see

TABLE 3 | Correlations between each fall metric, shown in the upper left of each sub table, and each significant gait, behavioral, and imaging measure.

	Gait			**Behavioral**			**Imaging**			**All**		
Fear of falling	**Single predictor model**			**Single predictor model**			**Single predictor model**			**Multiple predictor model**		
	p	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**
Tap cadence DTH	–	–	–	0.02	−0.04	−0.08, −0.01	–	–	–	0.004	−0.04	−0.08, −0.02
L SMA	–	–	–	–	–	–	0.02	−0.16	−0.29, −0.03	0.004	−0.18	−0.30, −0.06
Adjusted R^2		–			0.14			0.18			0.39	
F (for ΔR^2)		–			5.71			6.21			8.45	
Falls in past year	**Single predictor model**			**Single predictor model**			**Single predictor model**			**Multiple predictor model**		
	p	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**
Gait dual step length	0.02	−0.06	−0.10, −0.02	–	–	–	–	–	–	0.12	−0.04	−0.09, 0.01
Tap cadence DTH	–	–	–	0.03	−0.03	−0.06, −0.003	–	–	–	0.02	−0.04	−0.07, −0.01
R sup parietal cortex	–	–	–	–	–	–	0.02	−0.05	−0.10, −0.01	0.05	−0.04	−0.08, 0.001
Adjusted R^2		0.13			0.11			0.14			0.29	
F (for ΔR^2)		5.42			4.78			5.84			4.88	
Total number of falls	**Single predictor model**			**Single predictor model**			**Single predictor model**			**Multiple predictor model**		
	p	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**
Gait dual step length	0.02	−0.10	−0.18, −0.02	–	–	–	–	–	–	0.03	−0.08	−0.16, −0.01
R inf parietal cortex	–	–	–	–	–	–	0.02	−0.11	−0.20, −0.02	0.04	−0.09	−0.17, −0.01
Adjusted R^2		0.15			–			0.15			0.25	
F (for ΔR^2)		6.11			–			6.01			5.90	
Faller/non-faller (binary)	**Single predictor model**			**Single predictor model**			**Single predictor model**			**Multiple predictor model**		
	p	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***	**β**	**95% CI**
BY trials reaction time DTH	–	–	–	0.04	−0.008	−0.01, −0.001	–	–	–	0.02	−0.01	−0.01, −0.001
R DPMC3	–	–	–	–	–	–	0.04	−0.03	−0.05, −0.001	0.03	−0.03	−0.05, −0.004
Adjusted R^2		–			0.10			0.10			0.22	
F (for ΔR^2)		–			4.33			4.53			5.17	

Measures listed have a significant correlation ($p < 0.05$) with the fall measure in a single predictor model and are therefore entered into the multiple predictor model shown in the far right column. P-values (p), regression coefficients (β), and 95% confidence intervals (CI) are shown for each model, in addition to the adjusted R^2 and F values. Empty sub tables represent a fall measure and a measurement domain that did not yield any significant predictors for each single predictor model. DTH, dual-task hit; L SMA, left supplementary motor area; R DPMC, right dorsal premotor cortex.

Muir-Hunter and Wittwer (2016)]. Our study extends these prior results that despite the collection of data from an additional dual task and an fMRI paradigm, these gait task measures remain independently powerful as predictors of fall profile.

Changes in performance and brain functioning between single- and dual-task conditions in a stationary dual task paradigm are well aligned to prior stationary dual-task studies as well. Reaction time in the dual task condition decreased, consistent with multiple dual-task studies (for a meta-analysis, see Verhaeghen et al. (2003). Overall levels of brain activation in task-relevant brain regions reduced in the dual task condition compared to the single task condition as in prior fMRI studies (Bürki et al., 2017), for both the single tapping task and the single AX-CPT task. Our results extend these prior dual-task findings by showing that these dual task decrements in brain functioning and performance are associated with fall profiles. Our research suggests that dual tasking may create a bottleneck in brain resources, as supported by our results that this cohort didn't seem to prioritize performance of one component of the dual task over the other. Cognitive bottlenecks would imply that the limited amount of attentional resources was spent in trying to do each task "well enough," resulting in performance deficits in each task instead of a perfect completion of one task. In addition, the ROIs that were a significant predictor of falls have been shown in previous literature to be associated with falls. The right superior parietal cortex is involved in spatial orientation, and has been linked with cognitive-motor dual tasking in previous research (Bürki et al., 2017). The left SMA contributes to movement control, and the DLPMC is associated with motor planning, both of which had a significant correlation with at least one fall variable. A decrease in activation in these areas may contribute to fall risk, since it indicates less attention is put into those specific areas that help with movement.

We did not see a significant correlation between most accuracy and reaction time dual task hits (see **Supplementary Table 3**). About half of the data seem to cluster along the y-axis, indicating a sacrifice in tapping consistency in order to preserve accuracy. The other half is situated in the upper right quadrant, which represents decrements in both tapping consistency and accuracy. This agrees with findings in a meta-analysis on dual-tasking in older adults (Verhaeghen et al., 2003), which showed that while younger adults will sacrifice either reaction time or accuracy in dual task conditions, older adults will often do the same but can also have decrements in both reaction time and accuracy at the same time. Our data suggests that in the context of our specific stationary dual task, most participants favored accuracy more than reaction time or tapping consistency.

The key strength of this study was its multimodality. Gait characteristics, behavioral measures, and brain activity were all calculated in the same group of individuals to employ each of them as indicators of the fall profile. The main weakness of the study is the self-reported nature of the fall measures, which have known limitations in terms of recall bias. The volunteer participant sample of this study is also not representative (and is not intended to be representative) of the general population, and therefore caution should be applied in extending the results to the general population. The cohort of only 31 participants represents a small sample size but had a relatively high number of fallers so that the study retained a good amount of statistical power. Future work should extend this data *via* longitudinal assessment of the neuroimaging, gait, and behavioral measures as well as assessment within different age ranges and more objective measurement of falls using wrist- or waist-worn devices.

Clinically, a robust, quick, and cheap method to detect falls is preferred. This study does not attempt to replace these methods, but illustrate that there is much information that can be extracted from brain functioning measurements and applied to fall prediction. Since gait, behavioral, and brain imaging measurements provide independent information on fall risk, future work will attempt to provide stratification of fall risk using these multiple measurements domains.

In conclusion, this study found that diverse indicators of gait performance, cognitive and motor performance during a cognitive-motor dual task, and brain functioning during the dual task were all independent correlates of fall profiles in a group of community-dwelling, cognitively-normal older adults.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Pennington Biomedical Research Center Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AV and OC: experimental design. KK, SP, JK, JB, AD, and RB: data collection. KK: data analysis. KK, JK, OC, AV, and JB: data interpretation. KK and OC: manuscript drafting. All authors contributed to editing the manuscript.

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SUPPLEMENTARY MATERIAL

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METHODS
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Gauging Working Memory Capacity From Differential Resting Brain Oscillations in Older Individuals With A Wearable Device

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Working memory is a core cognitive function and its deficits is one of the most common cognitive impairments. Reduced working memory capacity manifests as reduced accuracy in memory recall and prolonged speed of memory retrieval in older adults. Currently, the relationship between healthy older individuals' age-related changes in resting brain oscillations and their working memory capacity is not clear. Eyes-closed resting electroencephalogram (rEEG) is gaining momentum as a potential neuromarker of mild cognitive impairments. Wearable and wireless EEG headset measuring key electrophysiological brain signals during rest and a working memory task was utilized. This research's central hypothesis is that rEEG (e.g., eyes closed for 90 s) frequency and network features are surrogate markers for working memory capacity in healthy older adults. Forty-three older adults' memory performance (accuracy and reaction times), brain oscillations during rest, and inter-channel magnitude-squared coherence during rest were analyzed. We report that individuals with a lower memory retrieval accuracy showed significantly increased alpha and beta oscillations over the right parietal site. Yet, faster working memory retrieval was significantly correlated with increased delta and theta band powers over the left parietal sites. In addition, significantly increased coherence between the left parietal site and the right frontal area is correlated with the faster speed in memory retrieval. The frontal and parietal dynamics of resting EEG is associated with the "*accuracy and speed trade-off*" during working memory in healthy older adults. Our results suggest that rEEG brain oscillations at local and distant neural circuits are surrogates of working memory retrieval's accuracy and processing speed. Our current findings further indicate that rEEG frequency and coherence features recorded by wearable headsets and a brief resting and task protocol are potential biomarkers for working memory capacity. Additionally, wearable headsets are useful for fast screening of cognitive impairment risk.

Keywords: working memory, EEG, resting-state, mild cognitive impairment, coherence analysis

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INTRODUCTION

Visual working memory plays pivotal roles in many daily goal-directed activities, such as searching for a car in a parking lot or driving. For an individual to find the right car, one must keep task-relevant information (e.g., the color of the car) in mind as a memory target, while rejecting non-target information. The task holds true while driving, where one needs to survey the mirror for the surroundings while the car is in motion. Retrieval accuracy and speed are essential memory performance measures that reflect working memory capacity as temporary storage of information and later manipulation (Hollingworth and Beck, 2016). There are age-related changes that occur in working memory, such as slowed neural processing speed or reaction times (Wang et al., 2011).

Recent advances in machine learning algorithms and wireless technology have allowed for wearable EEGs to gain renewed traction as a means to measure brain activity (Jiang et al., 2017; Abiri et al., 2019b). EEG signals can provide information about the oscillatory activity and brain functional connectivity across long-range brain networks (Abiri et al., 2019b). Study of resting-state and task-induced non-random patterns of intrinsic brain activities brings about major advantages such as being non-invasive, lower-cost, and portable (Noh et al., 2018; Guran et al., 2019; Mapelli and Özkurt, 2019; Nyhus et al., 2019; Román-López et al., 2019). For instance, analyzing event-related potentials (ERPs), which is the averaged EEG brain response onset to a psychological event (e.g., attentional vigilance or memory retrieval), offers a brain imaging technique to gauge cognitive processes (McBride et al., 2012; Li et al., 2017; Abiri et al., 2019a; Borhani et al., 2019; Jiang et al., 2021)1). Additionally wearable EEGs allows for the recording brainwaves during rest without additional instruments to induce a series of stimuli, i.e., similar to ERP-like experiments, which may induce less fatigue and anxiety in participants. Although there are plenty of advantages there are also disadvantages to wearable EEGs. One such disadvantage is that wearable EEGs are more susceptible to excessive body movement that can cause channel noise and artifacts in the recorded EEG signals, which can negatively impact the quality of the task-induced signals.

A variety of studies have examined the characteristics of resting-state measures to explain the neurophysiology of various diseases, such as migraines and Alzheimer's disease (AD), which further highlights the utility and quality of information obtained from rEEG measurements (Cao et al., 2016; Cassani et al., 2018). The dominant brain oscillation in awake resting-state is a low alpha oscillation (8–10 Hz), which is related to global attention. An overall increase in the low alpha power during the eyes-closed compared to the eyes-opened resting would be easily depictable. The increase is mostly manifested over parietal and occipital areas (Barry and De Blasio, 2017). Increased alpha positively correlates with higher accuracy and faster reaction times during verbal recognition tasks (Zunini et al., 2013). Resting eyes-closed EEG rhythms (e.g., posterior alpha and delta) change in pathological aging as a function of the global cognitive level (Babiloni et al., 2006). The higher alpha (10–13 Hz) is mostly correlated with sensorimotor and semantic processing related brain activities (Babiloni et al., 2010). Jeong (2004) studied brain oscillations during rest among normal aging individuals and individuals with dementia. They discovered increased delta (1–4 Hz) and theta (4–8 Hz) and decreased beta power among individuals with dementia when compared to the normal aging group. In regard to theta, Rossini et al. (2007) revealed that individuals with cerebrovascular dementia may have an overall higher theta oscillation when compared to individuals with AD.

In addition to diseased states, rEEG has been linked to cognitive performance in healthy normal adults. Analyzing resting-state EEG (rEEG) during eyes-open, a significant positive correlation between delta power captured over the left frontal and temporal regions and reaction times were observed among healthy adult participants. Additionally, higher frontal and parietal alpha correspond to lower accuracy and higher inter-trial variability of reaction times (Torkamani-Azar et al., 2020). Individuals with a lower working memory capacity have also shown larger changes between delta/theta ratios in rEEG with eyes open and eyes closed. The changes were also identified in the right posterior frontal and parietal cortices (Heister et al., 2013). Increased beta and gamma power over the right temporal and parietal areas during eyes-closed rEEG were positively correlated with second language acquisition (Prat et al., 2016), which is an indicator of working memory capacity. EEG signals' high temporal resolution allows the evaluation of functional connectivity by estimating coupling between different pairs of independent signal sources or different electrodes across frequency bands. The connectivity can capture relationships between different brain areas, providing valuable information to discover novel neuromarkers. Magnitude-squared coherence is a normalized measure of co-activation and temporal synchronization in the spectral domain between pairs of sources, or electrodes, representing functional coupling. The measure calculated by fast Fourier Transform, illustrating how information is processed during motor and cognitive processing between the active regions.

There is a network of brain regions subserving working memory functions. Older adults with cognitive impairment and preclinical AD pathologies show network connectivity changes (blood-oxygen level dependent signals at different brain regions) subserving working memory functions (Jiang et al., 2016). Thus, coherence during resting-state is a potential neuromarker of different neurodegenerative diseases. Babiloni et al. (2009) investigated the coherence network during resting between normal aging, MCI, and AD cohorts. The study revealed that AD cohorts have higher coherence over delta band and lower coherence over alpha oscillations compared to MCI and normal peers. Using magnetoencephalography (MEG) brain imaging, Stam and Van Dijk (2002) indicated a decrease in total brain synchronization in the beta and gamma bands among AD cohorts compared to normal aging cohorts. Bosboom et al. (2009) demonstrated that dementia in individuals with Parkinson's disease is positively correlated with a decrease of alpha frontotemporal coherence and a drop of local gamma oscillation during rest. Decrease of frontoparietal and interhemispheric coherence in the delta and alpha bands during rest in individuals

with AD has also been depicted (Sankari et al., 2011). Overall, a decrease in the measure of coherence in individuals with AD is often associated with damage in the cholinergic system and its interactions with the intrinsic neurotransmitters' excitation and inhibitions along pathways that connecting the frontal area to other brain regions, including temporal, occipital, and parietal areas (Scarr et al., 2013). The increase of coherence between the temporal region and other brain regions is considered a compensatory mechanism to tackle a decline in the coherence between other regions (Demirtaş et al., 2017).

Using a wireless headset with a 15 min resting and task protocol, we test the hypothesis that individuals with a higher working memory capacity show significant correlations with specific brain oscillations at local and distant neural circuits. Specifically, we explore relations between mean performance (i.e., reaction times and accuracy) of the Bluegrass memory task and the various rhythms during eyes-closed rEEG. Additionally, the connectivity between pairs of EEG electrodes in all oscillation bands were explored using magnitude-squared coherence between two EEG sites. The neural correlates of rEEG that correspond to task-dependent performance scores were measured in older adults with normal cognition. This research aimed to examine easy-to-use EEG markers to identify preclinical risk for cognitive impairments. The identification of these correlates is the first step toward early detection of preclinical changes in the brain that affects working memory (core cognitive functions). Early identification may allow for early intervention and prolongation of the maximal cognitive functioning in rapid-growing older populations.

MATERIALS AND METHODS

Human Participants

Using identical Bluegrass memory protocol, 43 community-dwelling older adults (age $\geq$ 60 years) including 20 males [median = 68 years, interquartile range (IQR) = 5.5], and 23 females (median = 72 years, IQR = 10) were recruited. All participants were tested at either the University of Kentucky (United Kingdom) Alzheimer's Disease (AD) Center (United Kingdom-ADC) or Aging, Brain, and Cognition Laboratory, at the Department of Behavioral Science, College of Medicine. The average age of participants was 71.6 $\pm$ 7.0 (min: 60, max: 91). Participants had a normal or corrected-to-normal vision and were not under the influence of any drowsiness-inducing or cognitive enhancing medication. Participants were all native English speakers, and mostly right-handed (6 were left-handed). To balance dominate hand bias (faster reaction times) within each participant, the index finger of the dominated hand was used to indicate memory target in the first 5 min of the task (**Figure 1**). Then the person's non-dominate hand was used to indicate memory target in the second 5-min of the task.

The experimental protocols were approved by the Institutional Review Board (IRB) of the University of Kentucky, Lexington, KY, United States. All participants provided signed informed consent in accordance with United Kingdom IRB. In other words, the recordings were carried out under the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Wireless EEG Signal Acquisition and the Experimental Procedure

Neural data collections were performed in a quiet and dimly lit room. A water-hydrated, portable, and wireless EEG headset, Emotiv EPOC+, was used for recording all EEG signals. The headset has 14 channels over AF3, F7, F3, FC5, T7, P7, O1, O2, P8, T8, FC6, F4, F8, and AF4 according to 10–20 International Electrode Placement System, and collects brain electrical activities with a sampling rate of 128 Hz with 14 bits resolution. A low impedance ($<$10 KΩ) was maintained for all EEG electrodes during the experiments. EEG signals were electrically referenced using CMS/DRL references at the left/right mastoid (P3/P4). Participants were seated comfortably on a chair 50 cm from a 24-inch LCD monitor. Every participant completed a 60-s resting session with eyes open, followed by a 60-s resting session with eyes closed.

The experimental interface provided the participants with instruction and primed them with a count-down to initiate resting sessions. During the eyes opened condition, the participants were requested to stay relaxed and look straight at a fixed-in-time and pleasant scenery of the blue ocean and avoid mind wandering. During the eyes closed condition, participants were requested to stay relaxed and avoid falling asleep once resting-state sessions were completed, participants were instructed to prepare for a memory task. A delayed-match-to-sample (DMS) task was adopted to assess simultaneous visual matching ability and short-term visual recognition memory. This short-term memory task, which is a variant of the Sternberg memory-scanning task (Sternberg, 1969) has been well-studied and can modulate various cognitive processes including encoding, decision making, visuomotor selection, rehearsal, and retrieval. In this current version of the task, participants were instructed to memorize two sample images in 5 s. Two target images with a green border were initially presented for 3 s (initial memory encoding). After a jittered delay (1.6$\sim$1.8 s), a sequence of 12 test images, including target and distractor images, were serially presented.

The number of target and distractor images were fairly distributed in each trial. Each trial lasted approximately 40 s, which included a 3-s encoding and 1-s presentation of each test image, and a fixation jittered inter-trial of 1.6$\sim$1.8 s in between (see **Figure 1**). Stimuli included two-dimensional black and white pictures of familiar objects taken from Snodgrass and Vanderwart (1980). Each picture was presented with a black background in an area of 8.3 cm $\times$ 5.8 cm. All stimuli were presented on a high-resolution color monitor with a 60 Hz refresh rate. Stimuli were presented at a 65-cm visual distance and a visual angle of approximately seven degrees. Test images were normalized across retrieval status (i.e., target matching or non-matching) for image familiarity and image complexity.

All participants practiced two sample trials prior to starting the memory task. The task was performed in two blocks of eight trials each. After presenting each picture, participants were

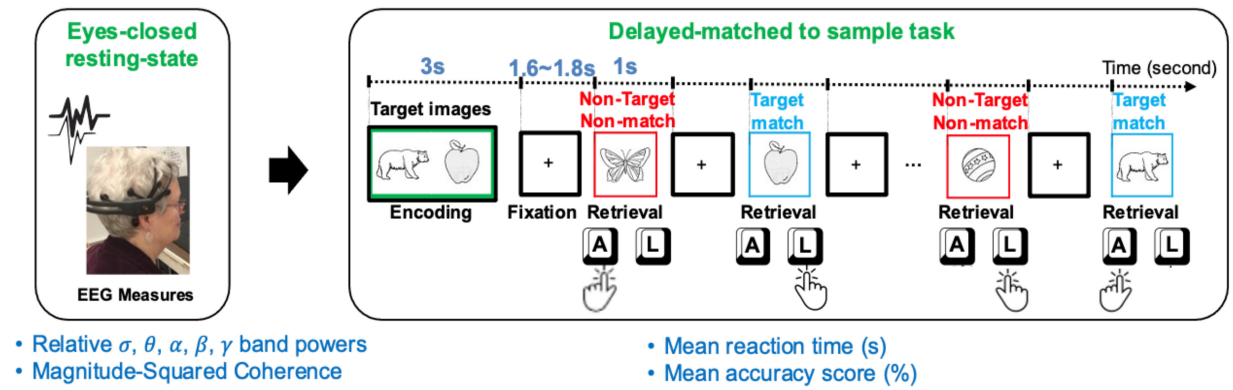

FIGURE 1 | A schematic of the resting EEG (rEEG) and Bluegrass memory protocol in a right-hand dominant human participant. The participants' eyes-opened resting-state followed by an eyes-closed resting-state followed by a memory task (a modified, delayed match-to-sample paradigm). The task includes encoding two target images in mind, then responding with the right index to indicate the memory target match (pressing "L" key on the keyboard) and left index finger for non-target in a 5-min task. During the second 5-min testing block, the participant was asked to indicate a memory target match by using the left index finger (pressing "A" key on the keyboard).

asked to respond whether the image matched one of the test images by pressing either the "A" or "L" key. During the first block, the participants responded to the match target images based on their dominant hand. For example, a right-handed individual would press the "L" key for a match and the "A" key for a non-match. The keys would be reversed for a left-handed individual- the match key would be the "A" key, and the non-match would be the "L" key. Once the first block was completed, participants were reinstructed for the second block. The second block required the match response to be from the non-dominant hand. Overall, the Bluegrass resting and memory protocol lasted approximately 15 min.

Newly Developed Experimental Software for Sharing

To eliminate the need for commercial software, a free PsychoPy3 builder and coder software (Brooks, 2019; Peirce et al., 2019) were utilized in the design and to present visual stimuli in a timely synchronous manner. The software suite is a version of the Psychopy python library used to develop, measure, and deliver different behavioral experiments. Behavioral responses in terms of key presses along with the continuous EEG signals were recorded during the experiment. Lab Streaming Layer (LSL) (Kothe, 2014) was used to synchronize behavioral and neural responses and record them into a single file. Researchers also developed and publicly shared a low-latency pipeline in Java programming language to parse in the real-time measured EEG data from the Emotiv EPOC+ application development interface (API) and parse out in LSL (O'neil, 2019). App-LabRecorder (2019) software was utilized as a unified recorder to collect and save behavioral and neural time series in a single file with the .xdf file extension. XDF is an open-source and general-purpose file format designed in tandem with LSL and supports LSL protocol features. The file extension is compatible with both MATLAB and Python languages and provides a convenient data structure container to record various data modality streams, concurrently. Researchers have publicly shared the experimental setup under open-source terms and conditions for other researchers [Borhani (2019) for free sharing]. The developed platform with all its software dependencies were installed on a computer in the clinics at United Kingdom-ADC and on a workstation in Aging, Brain, and Cognition Laboratory, United Kingdom College of Medicine.

Data Analysis

Resting-State EEG Preprocessing and Frequency Analysis

The impedance between each electrode and scalp were kept below 10 KW. EEGLAB software library (Delorme and Makeig, 2004) was employed for preprocessing and artifact removal. EEG signals were re-referenced to the average and band-pass filtered between 0.5 and 46 Hz. EEG recordings were analyzed for eye blinks and muscle artifacts. The artifact subspace reconstruction (ASR) algorithm (Kothe and Makeig, 2013; Gabard-Durnam et al., 2018) was implemented in the EEGLAB software and employed to cope with channel noise and artifacts. ASR algorithm is an advanced method that allows the detection and reconstruction of noisy and artifactual chunks of EEG signals. Makoto's EEG preprocessing pipeline (Makoto, 2018), along with Pernet et al. (2020) recommendations for EEG processing, were followed to process the EEG signals. Channels that were flat for more than 5 s, or with abnormal high/low peaks were deleted and removed. ASR finds the cleanest part of data as the calibration data by applying a 0.5-s sliding window principal component analysis (PCA) on the continuous EEG data to classify principal components into high variance (20 standard deviations the calibration data) or normal variance. By detecting high variance chunks of signals, ASR reconstructs the high variance subspace using the normal calibration chunks. A minimum of 60 s from each participant's data after channel noise and artifact removal was kept for analysis.

A Fast Fourier Transform (FFT) on 4-s epochs of EEG yields a 0.25 Hz frequency resolution over the frequency span of 1–46 Hz.

A sliding window of 4-s window length with a 2-s overlap was used to minimize the effect of the windowing of FFT procedure. The band power in delta (d:1–4 Hz), theta (q: 4–8 Hz), alpha (a: 8–13 Hz), beta (b: 13–28 Hz), and gamma (g: 28–46 Hz) frequency bands from all 14 EEG electrodes were computed. The absolute band power in each frequency band as well as magnitude-squared inter-channel coherence is computed for the eyes-closed resting-state session.

EEG Coherence Analysis

An electrode-by-electrode analysis was conducted to correlate rEEG band powers and magnitude-squared coherence with the memory task's performance measures. The approach helped to identify correlations between band-delimited power in all electrodes and the memory task performance. Pearson correlations between EEG band power and task performance presented the relationship, and significant correlations depicted the participants' dominant pattern. The recorded EEG data were analyzed using custom scripts in the MATLAB language (MATLAB, 2019). For each electrode, the correlation between the participants' behavioral measures of the memory task (mean reaction time and mean accuracy score) and mean power bands in the delta (d:1–4 Hz), theta (q: 4–8 Hz), alpha (a: 8–13 Hz), beta (b: 13–28 Hz), and gamma (g: 28–46 Hz) frequency bands were calculated using Pearson correlation. Additionally, the correlations between the inter-electrode coherence network during resting-state and the and memory task performance measures were analyzed. MATLAB connected topoplot toolbox (Namburi, 2020) was used to illustrate pairs of inter-channel coherence with a significant correlation between resting-state and task performance measures. To explore connectivity between all pairs of EEG electrodes in all oscillation bands, the magnitude-squared coherence was used as the connectivity estimation metric obtained by:

$$Coh_{xy}(f) = \frac{|G_{xy}(f)|^2}{G_{xx}(f).G_{yy}(f)} \quad (1)$$

Where G_{xx} and G_{yy} are autospectral density of channels x and y and G_{xy} is the cross-spectral density between EEG signals on channel x and channel y. Autospectral and cross-spectral density are functions of frequency. The magnitude coherence between two channels is an estimate showing the predictability of information from one channel using the other channel's data. We identified the neural correlates of resting-state EEG that correspond to task-dependent behavioral scores (see **Figure 1**) in a population of aging adults with normal cognition.

RESULTS

Using a wireless EEG setup in both laboratory and clinics, an 11-min visual working memory task (modified delayed matched to sample) was used to investigate rEEG surrogates of memory performance. Every participant's reaction-time (seconds) and accuracy score (correct percentage) during the working memory task were analyzed. Furthermore, correlations between the behavioral measures and neural measures were conducted.

Behavioral Results

Accuracy of Working Memory Retrieval

The mean and standard deviation (SD) of the older participants' percentage accuracy in correctly identifying the memory target and non-target stimuli were shown in **Figure 2**. The mean (SD) accuracy of the correct responses to target and non-target stimuli are 91.56 (5.02)% and 91.93 (5.84)%, respectively.

Reaction Times of Working Memory Retrieval

Figure 3 shows the mean and standard deviation of the reaction times to memory target match and non-match. Mean (SD) of correct reaction times to target and non-target images were

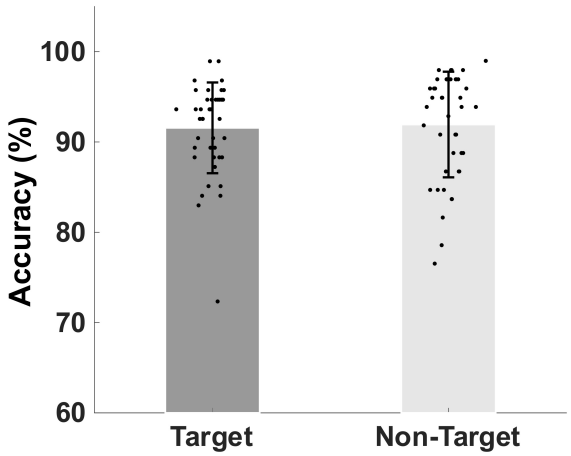

FIGURE 2 | Bar plot showing group mean percent and standard deviation of the mean accuracy of retrieval of target match and non-target, non-match visual stimuli.

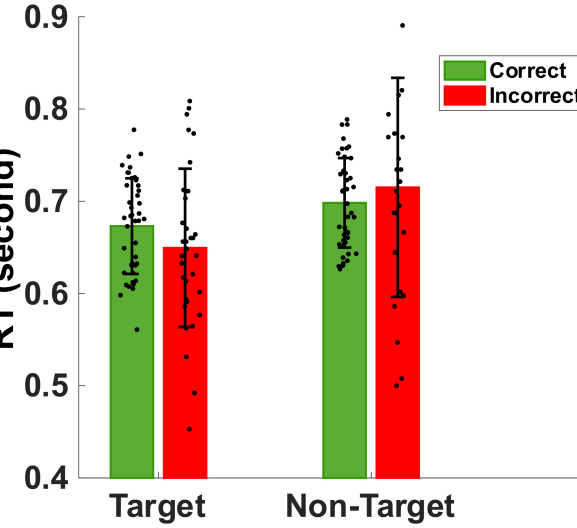

FIGURE 3 | Bar plot showing the group mean and standard deviation of the reaction times to target match and non-target distractors during correct (green) and incorrect (red) trials. The normal older adults were significantly faster in identifying memory targets than non-targets ($p < 0.05$).

673 (51) ms and 698 (48) ms, respectively. The non-parametric Kruskal–Wallis significance test revealed a significantly faster correct reaction to target-match compared to non-match ($p < 0.05$). Also, we extracted the mean incorrect reaction time to target and non-target (false alarm) stimuli. Mean (SD) of mean incorrect reaction time to target and non-target images are 649 (85) ms and 715 (118) ms, respectively. Non-parametric Kruskal-Wallis significance test revealed a significantly slower reaction to non-target non-matched (false alarm) images compared to target images ($p < 0.05$).

Sex Differences in Accuracy and Reaction Times

The male group (n = 20, median = 68, IQR = 5.5) were relatively younger than the female group (n = 23, median = 72, IQR = 10). Unsurprisingly, males showed a relatively higher accuracy (mean = 92.34%, SD = 4.32%) and faster reaction times (mean = 677 ms, SD = 51) (see **Figure 4**) compared to those of older females (accuracy: mean = 91.28%, SD = 4.97%, RT: mean = 693 ms, SD = 43).

Resting-State Alpha Band During Eyes-Closed and Eyes-Open

As a test of quality reassurance of our EEG data, we analyzed the eyes-closed and eyes-opened alpha bands because increased alpha activity under eyes closed is well established in the literature (as described in the introduction). We examined the group average and the group standard deviation of the alpha band power over the occipital sites (O1, O2) during resting-state (see **Figure 5**). The group mean ± SD during the resting-state eyes-closed is 38.80 ± 4.32 ($\mu V^2/Hz$) and during resting-state eyes-open is 35.65 ± 3.23 ($\mu V^2/Hz$). Using non-parametric Kruskal-Wallis significance test, the average occipital alpha during the resting-state eyes close is significantly larger than the eyes-open ($p = 3.6 \times 10^{-4}$). **Figure 6** illustrates average power spectral density during resting-state eyes opened and eyes closed and the corresponding topographic distribution of the alpha frequency spectrum (8–13 Hz), showing the increased occipital alpha during eyes-closed compared to eyes-open resting-state EEG.

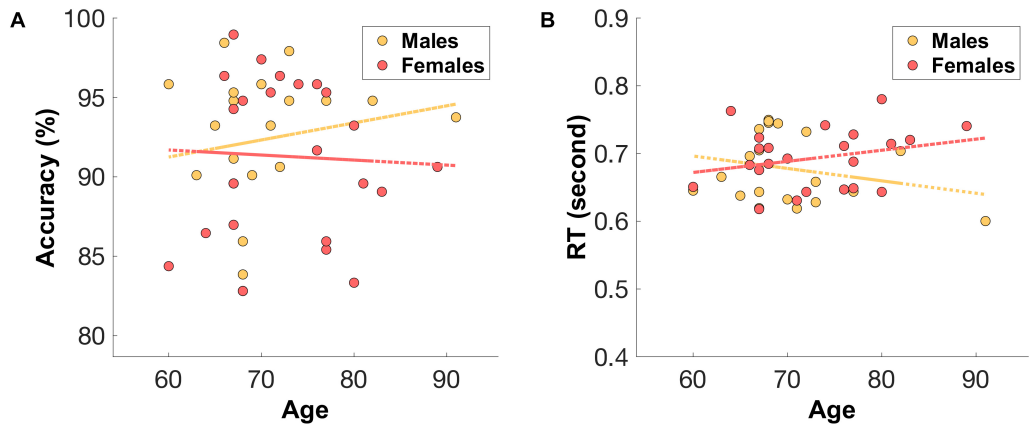

FIGURE 4 | The distribution of **(A)** mean accuracy score and **(B)** mean reaction time (RT) across age for both male (orange) and female (red) groups.

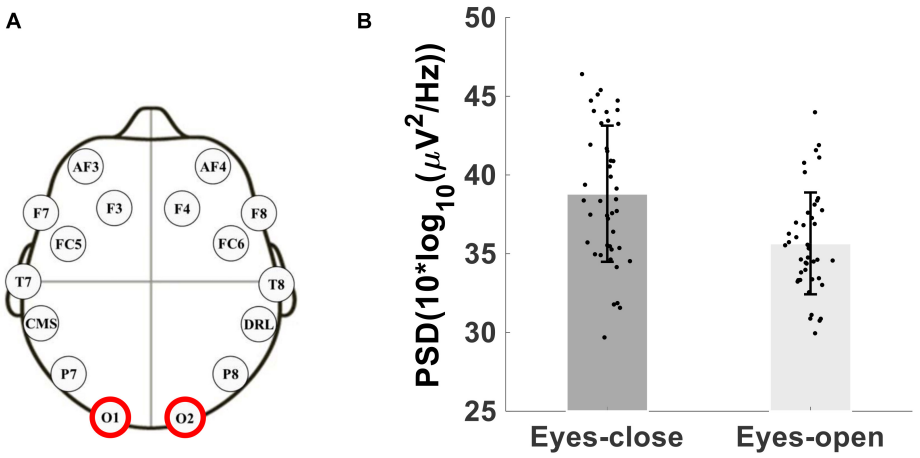

FIGURE 5 | (A) Montage of the 14-channels wireless EEG headset, **(B)** Bar plot showing the group average and standard deviation of the alpha band power over occipital sites (O1 and O2 electrodes) during eyes-open and eyes-closed.

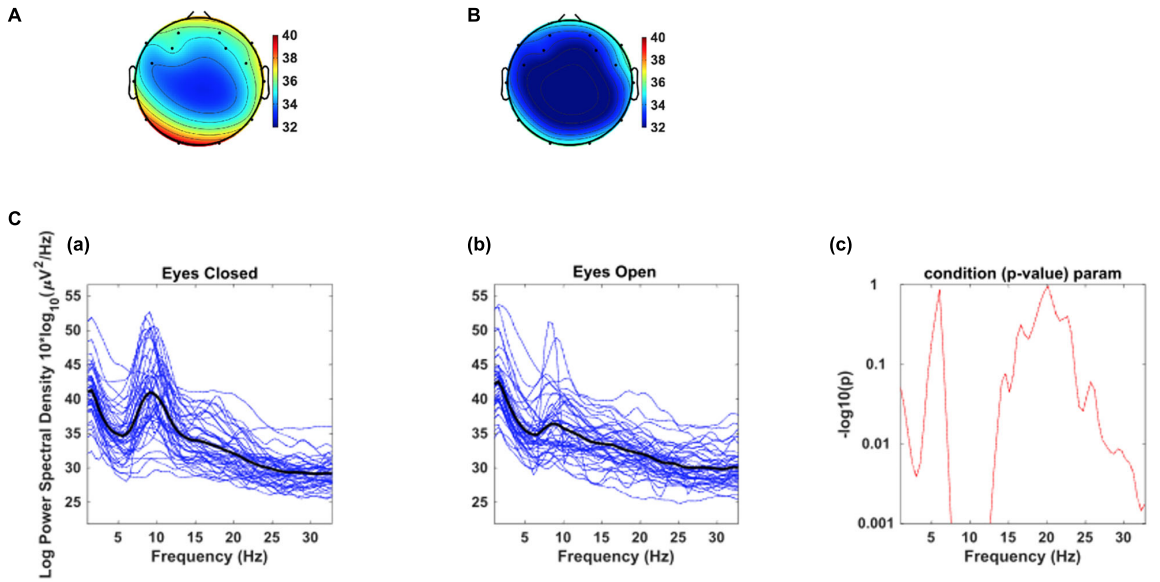

FIGURE 6 | The corresponding dorsal view topographic distribution of the alpha wave (8–13 Hz), during resting-state **(A)** eyes-closed and **(B)** eyes-open (Top panel). C. Average power spectral density at combined occipital sites (O1 & O2) during eyes-closed **(A)** and eyes-open **(B)**; The black color curves show the average occipital power spectral density of all participants, and the blue curves show individuals' power spectral density. **(C)** the point by point confidence level shown by p-value in the older adults.

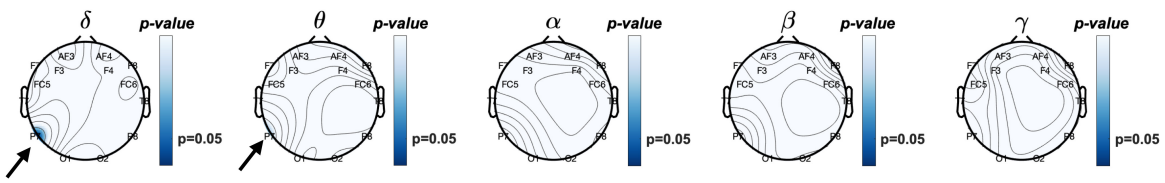

FIGURE 7 | Topographical plot of Pearson correlations between resting EEG (Eyes-closed) relative frequency band power and mean reaction times during working memory task. Significant negative correlation was observed at the P7 (left parieto-occipital site) at delta (δ) frequency band ($p < 0.05$) and approaching significant ($p = 0.068$) theta (θ) band power. In other words, increased δ and θ frequencies power observed in the left posterior brain region is correlated with faster reaction times. Reaction times did not correlate with higher frequency band power, i.e., α, β, nor γ.

Eyes-Closed Resting-State EEG and Task-Related Behavior

rEEG and Reaction Times

Since eyes-closed rEEG is gaining momentum as easy biomarker for cognitive decline, we examined Pearson correlations between rEEG relative frequency band power recorded during the resting-state eyes-closed session before the memory task and mean behavioral results during the working memory task. **Figure 7** show the topographical distribution of the Pearson correlations between eyes closed rEEG relative frequency band power and reaction times during the memory task. The significant correlations between rEEG power spectral density in each frequency band on 14 EEG sites and the individuals' performance in the working memory task were investigated. A significant negative correlation was found between individuals' mean reaction times during the memory task and the delta band power ($\rho\,(43) = -0.31,\ p < 0.05$). The correlation is mostly focused over the left parietal site over the P7 EEG electrode.

With approaching significance, higher activities in the theta band over the left parietal correlates with faster reaction time ($\rho\,(43) = -0.28,\ p = 0.07$). As shown in **Table 1**, brainwave EEG during resting did not correlate with mean reaction times in the higher frequency bands in alpha, beta, and gamma bands.

rEEG and Accuracy

Next, we explored a potential rEEG neuromarker of individuals' mean accuracy scores (percentage of correct responses) during a short-term memory task. **Figure 8** reveals the topographical distribution of Pearson correlations between the eyes-closed rEEG and mean percent accuracy during the memory task.

TABLE 1 | Correlations between eyes-closed resting-state EEG and mean reaction time.

Frequency band (Hz)	Electrode (location)	Pearson correlation (ρ)	*p*
delta (1–4 Hz)	P7 (left parieto-occipital)	−0.31	0.042*
theta (4–8 Hz)	P7 (left parieto-occipital)	−0.28	0.068

**indicates significant p value at the 0.05 level.*

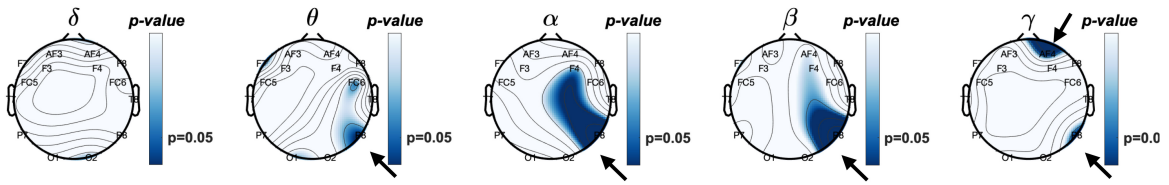

FIGURE 8 | Topographical plot of Pearson correlations between relative frequency band power of resting eyes-closed EEG and Accuracy (% correct) of the working memory task.

TABLE 2 | Correlations between eyes-closed resting-state EEG and mean accuracy score.

Frequency band (Hz)	Electrode (location)	Pearson correlation (ρ)	*p*
delta (1–4 Hz)	O2 (right occipital)	−0.29	0.058
theta (4–8 Hz)	F7 (left frontal)	−0.31	0.043*
	O1 (right occipital)	−0.28	0.064
	P8 (right parietal)	−0.31	0.041*
	FC6 (right frontocentral)	−0.29	0.059
alpha (8–13 Hz)	P8 (right parietal)	−0.39	0.0095**
	F4 (right frontal)	−0.28	0.068
beta (13–28 Hz)	P8 (right parietal)	−0.42	0.0054**
gamma (28–46 Hz)	P8 (right parietal)	−0.32	0.033*
	AF4 (right frontal)	−0.33	0.032*

**indicates significant p value at the 0.05 level; **indicates significant p < 0.01.*

A significant negative correlation ($\rho\,(43) = -0.42$, $p < 0.01$) was found between individuals' EEG frequency band power in the beta band over the right parietal site (P8 electrode).

Table 2 shows Pearson correlations and the significance level in terms of p-value for the EEG frequency bands and EEG electrodes. The significance level of correlations between eyes-closed resting-state and mean accuracy scores were lower than 0.05. The overall distribution of the Pearson correlations in all frequency bands and all EEG electrode sites suggests that lower brain activity over the right parietal site in alpha and beta bands as well as the right frontal site in the gamma band during eyes-closed resting-state was significantly correlated with higher ability to correctly distinguish between target and distractor images in a short-term memory task. Lower alpha and beta activities of rEEG over right parietal areas during eyes-closed were found to be indicators of higher accuracy score.

Magnitude-Squared Coherence of the Fronto-Parietal Sites Subserving Reaction Times

The coupling between brain sites within the delta, theta, alpha, beta, and gamma frequency bands based on the magnitude-squared coherence algorithm was explored. As demonstrated in **Figure 9**, significant correlations ($p < 0.05$) between paired eyes-closed rEEG magnitude-squared coherence in frequency band oscillations during and mean reaction time were extracted. Significant differences were mainly found in the alpha band in the EEG inter-channel coherence network. Increased alpha coherence between the right parietal and the left frontal sites correlates with increased reaction time.

Coherence of the rEEG at Parietal Sites in the Delta and Theta Bands Correlates With the Mean Percent Accuracy

As demonstrated in **Figure 10**, significant correlations ($p < 0.05$) between paired rEEG magnitude-squared coherence in oscillations during frequency band and mean accuracy score

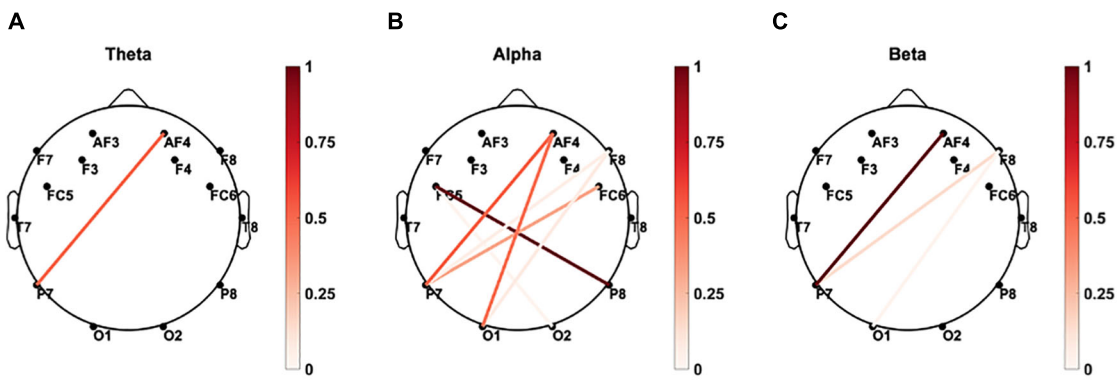

FIGURE 9 | Brain connectivity graphs showing the significant correlations between paired EEG channels' magnitude-squared coherence during the eye-closed resting-state and mean reaction time in **(A)** delta, **(B)** theta, **(C)** alpha, **(D)** beta, and **(E)** gamma bands. The color designates the level of correlation between paired coherence and the reaction times were significant ($p < 0.05$). Increased alpha coherence between the left frontal and right posterior sites correlates with increased reaction time.

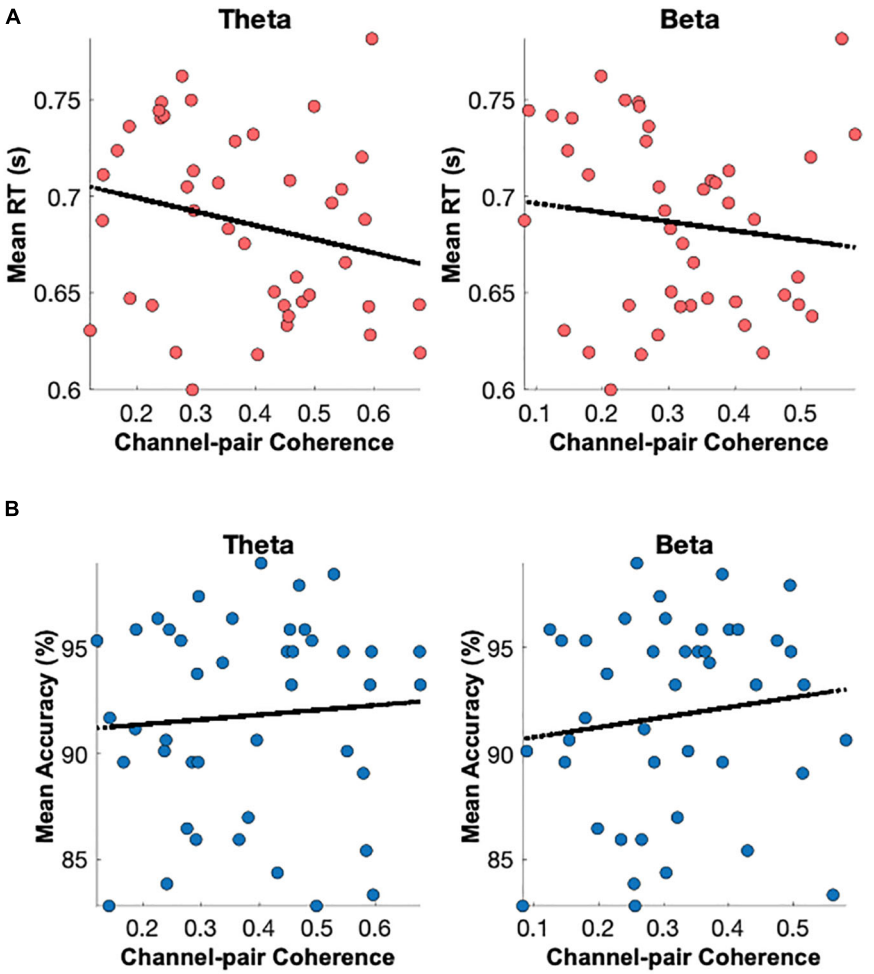

FIGURE 10 | Brain connectivity graphs showing the significant correlations between mean percent accuracy and paired rEEG channels' coherence during the eye-closed in **(A)** delta, **(B)** theta, **(C)** alpha, **(D)** beta, and **(E)** gamma bands. The color designates the level of correlation between all paired coherence and the corresponding measure where the observed correlation was significant ($p < 0.05$). Significant increased of frontal delta and theta coherence correlates with better memory accuracy.

were extracted. Significant correlations were found in the theta and delta bands for the rEEG inter-channel coherence network. Increased frontal contralateral coherence between frontal and temporal sites is positively correlated with increased mean accuracy scores during memory retrieval. No significant correlations were noted in the inter-channel coherence network in the beta band and mean accuracy score.

DISCUSSION

Summary of the Findings

We report several new findings to explore the clinical-friendly eyes-closed rEEG and healthy older individuals' working memory capacity. First, working memory retrievals' accuracy is correlated with higher frequency brain oscillations (beta and alpha) at local and distant neural circuits in the right hemisphere support. Second, faster memory retrieval is significantly correlated with increased delta and theta band powers over the left parietal sites. Also, increased coherence between the right parietal site and the left frontal area is associated with slowed memory retrieval speed.

Accuracy of Working Memory Retrievals and Gamma, Beta, and Alpha

Before we test our hypothesis, we analyzed memory performance data and alpha eyes closed and open data to make sure the quality of our performance and EEG data. We found that accuracy is approximately 91% to correctly identify one of the two memory targets and non-target distractors in the current working memory task in older adults. In a slightly easier version of the task (only one memory target), the accuracy was 92% and 97% (Lawson et al., 2007). Eyes-closed (EC) evoked larger alpha powers than eyes open (EO) has been well documented in the literature. Interestingly, the difference between EC and EO are reduced in patients with MCI and AD (Wan et al., 2019). The results from the current research are consistent with previous literature of rEEG in healthy older adults.

We report the significant correlations between memory retrieval accuracy and rEEG band power. Specifically, increased alpha and beta bands over the right parietal and gamma in the right frontal sites, were significantly correlated with poorer accuracy during the memory task. The right parietal site within alpha and beta bands showed even a higher relationship with the mean accuracy compared to the gamma band.

Reaction Times and Delta/Theta Band Powers

Interestingly, memory retrieval of non-match distractor objects took longer than target objects (see **Figure 3**). Pearson correlation was analyzed between neural oscillations in different frequency bands recorded with a wearable EEG headset during eyes-closed resting-state and the participants' mean reaction time and mean accuracy score during a delayed visual target matching task. The results suggest that rEEG in specific brain sites and specific rhythms are significantly correlated with working memory performance measures. Researchers discovered that the analysis of EEG signals during resting and conscious state of mind carries a predictive utility to tell apart behavioral performance associated with short-term memory performance. Results of significant correlations of rEEG band powers and individuals' mean reaction time indicate that a faster reaction in target matching memory task to significantly correlates with higher delta and theta rhythms over left parietal sites (**Figure 7**). Additionally, we discovered that rEEG over right parietal and right frontal sites carried valuable information about the mean accuracy of target recognition in the memory task. Lower brain activities in alpha, beta, and gamma bands over the right parietal region correlate well with a higher accuracy score. These results suggest that rEEG over the parietal can be a potential biomarker, reflecting memory retrieval accuracy among normal aging adults.

The current findings are consistent with the literature that higher theta and lower beta, which results in higher theta/beta ratio before, is an indicator of memory capacity. Heister et al. (2013) studied neural associates of working memory during resting-state captured by MEG signals, which found that right frontal and parietal cortex delta/theta power were inversely correlated with three-back working memory performance. Those results demonstrated that individuals with poor accuracy in working memory showed larger increases in right posterior frontal and parietal delta/theta in resting-state condition. An individual's working memory also requires input from attention. A new study highlighted the predictive utility of resting-state EEG and investigated neural correlates of vigilance score and response time during varying-duration sessions of sustained attention to response task (SART) (Torkamani-Azar et al., 2020). The results indicated an increase in the left central and temporal gamma, and upper beta during rest predicts slower reaction time.

Selective Neural Communications Within the Working Memory Network *via* Frequency Oscillations and Coherence

The nature of neural coding in mammalian brains, which may support selective communication, are not fully understood. Research has postulated that the structure of oscillations varies in time and space according to behavioral state. Multiplexing implemented through periodic modulation of firing-rate population codes enables flexible reconfiguration of effective connectivity. Memory retrieval is a dynamic process continuously regulated by both synaptic and intrinsic neural mechanisms, e.g., intrinsic excitability, synaptic plasticity, and interactions among brain areas (Chen et al., 2020). The findings suggest that neural dynamics underlying accuracy are different from those undeserving memory retrieval speed.

By analyzing spectral coherence between paired EEG sites, we reported increased connectivity between resting EEG sites and working memory accuracy and reaction times. Literature has suggested the significance of coherence between frontal and posterior brain areas as an indicator of brain overall cognitive function. In a comprehensive review of the literature (Babiloni et al., 2016), changes in the connectivity network between different brain areas, including the frontal and parietal areas, have been observed among people with MCI and AD. The research has demonstrated that the coherence between frontoparietal regions in the theta band would correlate with the working memory performance-related measures on the Digit Span (Tóth et al., 2012). Fleck et al. (2016) studied resting-state EEG in search of a neuromarker of cognitive decline, and they observed a positive correlation between the delta and the beta coherence within the frontal and posterior regions and performance on measures of memory and executive function in older adults.

Implications and Limitations

Findings suggest an increased resting eyes-closed delta and theta bands at the left parietal site are associated with faster speed, while increased alpha and delta at right parietal site is associated with reduced accuracy during working memory. Increased coherence between the right parietal and the left frontal sites correlates with slowed reaction time. The frontal and posterior dynamics of resting EEG is associated with the "*accuracy and speed trade-off*" during working memory in healthy older adults.

Importantly, the wireless headset is a beneficial, pre-screening tool utilized at physician offices before sophisticated biomarkers tests in various clinical populations. Deficits in working memory are common in amnestic mild cognitive impairment (MCI), an early stage of AD, and related dementia (ADRD). Currently, the AD biomarkers for early diagnosis of mild cognitive impairment are not only expensive to measure, but also involve invasive and time-consuming neuroimaging, laboratory examinations, and cognitive assessments. Depending on the degree of change, the quantitative analysis of cognitive processing changes may provide evidence of pathologic cognitive changes beyond age-related decline, MCI, and ADRD.

The study is a preliminary stage to test the utility of the resting-state EEG using a wearable headset as a pre-screening methodology for cognitive measures besides other well-established behavioral and cognitive questionnaires. There are both advantages and disadvantages to current rEEG approaches, which gauge working memory capacity. Currently, clinicians rely on well-established but time-consuming neuropsychological testing as a proxy to identify changes in brain

networks in the aging population. Different cognitive tasks such as working memory and selective attention have been used to enumerate the rate of cognitive decline measured by accuracy and reaction time, which has been shown to provide a reliable cognitive decline measure. Cognitive testing is less sensitive for the small changes in memory that accumulate overtime but can identify the changes' summation when the effect is significant. However, cognitive deficiency may not be appeared and diagnosed at the onset of AD, motivating the discovery of non-invasive and inexpensive-to-collect neuromarker, i.e., a neuromarker showing altered brain connectivity across interconnected networks of the brain.

A noted limitation in the present study was a small sample size (n = 43). As such, further research is necessary to understand the underlying neural mechanisms. Additionally, a follow-up study should include a longitudinal approach that includes a large number of healthy, aging adults. With the wearable and wireless EEG headset that is non-invasive and easy-to-use, the current study offers an affordable option for large-scale repeated recording in the clinics. Furthermore, research efforts should focus on evaluating EEG neuromarkers in patients with amnestic MCI, or those in dementia. Future investigation is needed to evaluate the associations and correlations of rEEG with other neural degenerative biomarkers such as A-beta, p-tau, or cortical atrophy in MRI scans. Overall, brief resting and task protocol using wearable EEG headset has demonstrated great potential for gauging working memory capacity, a sensitive and fast screening tool for cognitive impairment risk. Our current findings indicate the need for future large-scale validation to understand individual measurements of rEEG frequency and coherence features.

CONCLUSION

We report that the frontal and posterior dynamics of resting EEG is associated with the "*accuracy and speed trade-off*" during working memory in healthy older adults. The Bluegrass protocol recorded resting EEG oscillations under 3 min and likely to be used as fast surrogate markers for assessing individual working memory capacity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of the University of Kentucky, Lexington, KY, United States. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YJ, GJ, SB, and XZ designed the research. MK, KG, and GJ conducted the neuropsychological and EEG data collection and behavioral data analysis. SB, YJ, FY, and XZ performed the EEG and statistical analysis. SB, YJ, and XZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Correlation of Frontal Atrophy and CSF Tau Levels With Neuropsychiatric Symptoms in Patients With Cognitive Impairment: A Memory Clinic Experience

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Background: Behavioral and psychological symptoms of dementia (BPSD) are a distressful condition. We aimed to investigate the BPSD distribution in subjects with cognitive impairment, and the potential correlations between BPSD and neurodegeneration in terms of cerebrospinal fluid (CSF) tau and brain atrophy.

Methods: One-hundred patients with mild cognitive impairment (MCI) or dementia (Alzheimer's disease, AD; Lewy-body disease, LBD; frontotemporal dementia, FTD; vascular dementia, VD) underwent a complete diagnostic workup, including 3T-MRI and/or CT and CSF. Cortical atrophy was assessed with medial temporal atrophy (MTA), posterior atrophy (PA), and global cortical atrophy-frontal lobe (GCA-F) scales. BPSD were rated using the Neuropsychiatric Inventory (NPI), and BPSD clusters were defined according to the European Alzheimer Disease Consortium.

Results: Delusions, hallucinations, and psychosis cluster were differently distributed among the diagnostic groups ($p < 0.05$, $p < 0.001$, and $p < 0.05$), with LBD patients showing higher scores for hallucinations (vs. MCI, $p < 0.001$, and AD, $p < 0.05$) and psychosis cluster (vs. MCI, $p < 0.05$). In primary dementias, we found a negative correlation between NPI total score and tau levels ($p = 0.08$), confirmed by beta regression ($p < 0.01$), while a positive non-significant relationship was observed in MCI. Higher GCA-F scores were associated with delusions and apathy ($p < 0.05$, on both hemispheres) and hallucinations (left: $p < 0.01$, right: $p < 0.05$). GCA-F scores were positively correlated with psychosis cluster (right: $p < 0.05$), and agitation/aggression (left: $p < 0.05$). Conversely, nighttime disturbances were positively correlated with both GCA-F and MTA scores (left: $p < 0.01$; right: $p < 0.05$).

Abbreviations: AD, Alzheimer's disease; BPSD, behavioral and psychological symptoms of dementia; CSF, cerebrospinal fluid; CT, computed tomography; FTD, frontotemporal dementia; GCA-F, global cortical atrophy-frontal lobe; LBD, Lewy body dementia; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; MTA, medial temporal atrophy; NPI, Neuropsychiatric Inventory; PA, posterior atrophy.

Conclusion: Our results suggest that psychotic symptoms are significantly more represented in LBD patients and that CSF tau and frontal atrophy are associated with the occurrence and severity of BPSD in clinical practice. Longitudinal studies are however required to ascertain their actual predictive value.

Keywords: behavioral and psychological symptoms of dementia, neuropsychiatric inventory, CSF biomarkers, atrophy visual rating scales, cognitive impairment

INTRODUCTION

Dementia is a pathological condition with a strong impact on the quality of life of both patients and caregivers. In 2019 the World Health Organization estimated the global prevalence of dementia at around 50 million, with a trend to triple by 2050 (World Health Organization, 2019). The symptomatologic core of dementia consists of cognitive decline with a significant functional disability in daily life and progressive evolution (McKhann et al., 1984). Nonetheless, behavioral and psychological symptoms (BPSD) are a common feature affecting 98% of individuals with dementia (Phan et al., 2019), even in the early stages of disease (Di Iulio et al., 2010; Spalletta et al., 2012). These symptoms are usually referred to by the caregiver as the most critical and distressful aspects of the disease (McKeith and Cummings, 2005), and appear to be related to elevated disability, institutionalization, and death (Scarmeas et al., 2005). A recent study on a cohort of 181 subjects from the Alzheimer's Disease Neuroimaging Study (ADNI) confirmed an inverse correlation between BPSD and cognitive/functional outcomes in patients with Alzheimer's disease (AD), suggesting the importance of their early recognition to improve the disease management (Poulin et al., 2017). Confirming this, patients with amnestic mild cognitive impairment (MCI) and BPSD (in particular, apathy) had an almost sevenfold risk of AD progression (Palmer et al., 2010). Also, some BPSD display a good sensitivity and specificity, such as to have been included among the core or support diagnostic criteria of specific forms of dementia. This is the case of visual hallucinations and REM behavioral disorders in Lewy bodies dementia (LBD; McKeith et al., 2017), and disinhibition, apathy, aberrant motor behavior, and eating disorders in frontotemporal dementia (FTD; Rascovsky et al., 2011). It is thus essential, both for diagnosis and for management, to recognize these symptoms timely and track their evolution during the disease.

A possible link between BPSD patterns and profiles of cerebrospinal fluid (CSF) biomarkers has been often suggested, but conclusive data are still missing. In cognitively normal older adults, higher values of tau/Aβ42 were found to predict longitudinally greater increases of negative emotions, such as anxiety and depression (Babulal et al., 2016). In subjects with mild cognitive impairment (MCI), Ramakers and coll. observed a relationship between high *t*-tau levels and anxiety, and between low Aβ42 levels and agitation, irritability, and anxiety (Ramakers et al., 2013). Similarly, low Aβ42 levels were found to correlate with depression in another MCI population (Gonzales et al., 2018). In AD patients with mild-to-moderate dementia, high *t*-tau and *p*-tau levels were variously associated with different BPSD, in particular agitation and apathy (Skogseth et al., 2008; Bloniecki et al., 2014), and an inverse correlation between Aβ42 and aggressiveness was observed (Engelborghs et al., 2006).

A second relevant investigation field, based on neuroimaging, is the one focused on the research of possible links between morphological and functional cortical abnormalities and BPSD. Visual rating scales are a widespread and easy-to-use tool to assess atrophy, for both clinical and research purposes (Scheltens et al., 1992; Koedam et al., 2011; Van der Flier and Scheltens, 2018). In this regard, very little is known on the correlation among visual rating scales and BPSD: in AD, high medial temporal atrophy (MTA) scores were significantly associated with apathy and disinhibition (Garcia-Alberca et al., 2019), while posterior atrophy (PA) on the right hemisphere was associated to agitation and aggression (Hsu et al., 2015). Previous neuroimaging studies, assessing morphological, perfusion, and metabolic brain changes in AD patients, found that BPSD, such as delusions, apathy, and depression, were particularly associated with a frontal region involvement, predominantly of the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC; Boublay et al., 2016). Similarly, studies in subjects with MCI reported a link between apathy and hypoperfusion of the frontal, temporal, occipital lobes (Kazui et al., 2017) and inferior temporal and anterior cingulate atrophy (Guercio et al., 2015). Similar investigations were conducted also in non-AD dementias: in the behavioral variant of frontotemporal dementia (bvFTD), a close correlation was observed between disinhibition and atrophy of specific frontotemporal areas (ventromedial orbitofrontal, medial frontal, and anterior temporal lobe; Hornberger et al., 2011), while in Lewy body dementia (LBD), a dysfunction of both associative visual areas and limbic areas was found to be associated with the occurrence of hallucinations (Burghaus et al., 2012).

Given the variability of the evidence on BPSD so far available in the literature, this study aimed to expand the knowledge of the biological correlates of the neuropsychiatric symptoms, evaluating the impact of BPSD in different forms of cognitive impairment [MCI, AD, FTD, LBD, and Vascular Dementia (VD)], and searching for potential correlations between BPSD and CSF biomarkers and cortical visual rating scales.

MATERIALS AND METHODS

The study was designed and carried out at the IRCCS Mondino Foundation, with the collaboration of the University of Pavia, Italy. Recruitment started in June 2018 and was completed in February 2020.

All procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Participants or their legal representatives provided written informed consent to all the diagnostic procedures included in this study. No participant received financial compensation.

Participants

Participants were cognitively impaired patients undergoing diagnostic workup at the Behavioral Neurology Unit of the IRCCS Mondino Foundation. Inclusion criteria were: a diagnosis of MCI (amnestic or non-amnestic/single or multiple domains; Albert et al., 2011) or dementia; age between 50 and 90 years and an available informant with at least 10 h per week of contact with the patient. No limit of the severity of dementia was set, as long as the patient was able to perform a formal cognitive assessment. The vision and hearing acuity of patients were sufficient for compliance with testing procedures. Patients were excluded if they had a history of psychiatric disease or epilepsy, or any uncontrolled medical condition that could contribute to the subject's cognitive impairment (e.g., nephropathy, liver disease, brain tumor, alcohol or drug abuse, normal pressure hydrocephalus). None of the patients were receiving medications for dementia, such as cholinesterase inhibitors or antipsychotic drugs, at the time of the diagnostic workup. Previously, 10 patients had taken antipsychotic drugs, and 11 had been on cholinesterase inhibitors.

Study Design

The study was designed as a single-site cross-sectional study. Enrolled patients underwent complete clinical, neurological, and neuropsychological assessment, brain imaging (magnetic resonance imaging, MRI, or computed tomography CT), CSF collection (for the assay of Aβ42, total tau, and phospho-tau levels), and Neuropsychiatric Inventory (NPI) assessment. The neuropsychological examination included tests for global cognitive efficiency (Mini-Mental State Examination, MMSE), and for memory (Verbal Span, Digit Span, Corsi Test, 15 Item Memory Test, Story Recall Test, Rey Complex Figure delayed recall), logical and executive functioning (Raven's Colored Matrices, Frontal Assessment Battery), attention (Trail Making Test A/B, Attentive Matrices, Stroop Test), language (Semantic and Phonemic fluency tests) and visual-spatial perception (Rey Complex Figure copy). NPI assessment was performed asking the caregiver to indicate *via* the screening questions whether the patient had experienced any domain-related neuropsychiatric symptom over the previous month. If the screening questions were validated, the caregiver was then asked to provide a domain rating for frequency, severity, and level of distress, and the total domain score was the product of the ratings for frequency and severity (Cummings et al., 1994). Four main NPI clusters (hyperactive behaviors, psychosis, affective behaviors, and apathy) were defined according to Aalten et al. (2008). "Hyperactive behaviors" cluster included agitation, euphoria, disinhibition, irritability, aberrant motor behavior, and night-time behavior disturbances; "psychosis" cluster included delusions and hallucinations; "affective behaviors" cluster included anxiety and depression; finally, "apathy" cluster included apathy and appetite/eating abnormalities.

Once the diagnostic workup was completed, patients were classified into two syndromic categories, MCI, and dementia, and the latter patients received an appropriate etiological diagnosis according to the most recent diagnostic criteria. MCI subjects were diagnosed according to NIA-AA criteria (Albert et al., 2011), and had clinical dementia rating (Morris, 1993) (CDR) = 0.5. Subjects with dementia received an etiological diagnosis of typical AD (Dubois et al., 2014), a behavioral variant of frontotemporal dementia (bvFTD; Rascovsky et al., 2011), Lewy body dementia (LBD; McKeith et al., 2017), or VD (Román et al., 1993), and had CDR $\geqq$1. All patients with non-vascular dementia had a score <4 on the Modified Hachinski Ischemic Scale (Hachinski et al., 1975). Despite the advanced diagnostic workup including morphological and CSF biomarkers, 6 demented patients could not receive an etiological diagnosis with high confidence, and therefore were classified into a separate group, as not otherwise specified dementias (Dem NOS).

Neuroimaging and CSF

MRI and CT scans were acquired at the Neuroradiology Unit of IRCCS Mondino Foundation. For this study, we analyzed 76 3D T1-weighted sequences acquired with Magnetom Skyra 3T (Siemens Healthcare), and 15 tomograms acquired with Somatome Perspective CT (Siemens Healthcare). MTA, PA, and the global cortical atrophy-frontal (GCA-F) scales were rated on 3D T1-weighted MR images, according to the original descriptions (Scheltens et al., 1992; Pasquier et al., 1996; Koedam et al., 2011), on both hemispheres. The MTA scale assesses the width of the choroid fissure and the temporal horn, as well as the height of the hippocampus; the PA scale assesses the width of the posterior cingulate- and parieto-occipital sulci, and the atrophy of the parietal lobe and precuneus; the GCA-F evaluates the severity of the atrophy of the frontal lobes. On CT images, only the MTA scale was rated. Visual ratings were collegially performed by two raters with more than 2 years of experience in the visual rating and over 900 neuroimages assessed from the LANE dataset (Cotta Ramusino et al., 2019). The raters were blind to diagnosis and CSF profile. Nine scans did not undergo visual rating due to the presence of artifacts or excess motion.

In 87 participants, a lumbar puncture was performed at the level of the L3/L4 or L4/L5 intervertebral space, according to the standard procedure used for patients with cognitive disorders in our clinic. CSF samples were centrifuged for 10 min at 1,800 *g* at 4°C within 3 h of collection. The samples were then divided into aliquots of 0.5 ml in polypropylene tubes and stored at −80°C. Measurement of CSF Aβ42, *t*-tau, and *p*-tau was performed using chemiluminescence enzyme immunoassay (Lumipulse G600II, Fujirebio); only for 14 participants, Aβ40 was available due to the relatively recent introduction of this assay in our laboratory. Biomarker profile was considered suggestive of AD pathology if Aβ42 < 599 pg/ml, *t*-tau > 404 pg/ml and *p*-tau > 56.5 pg/ml, or Aβ42/*t*-tau < 1.27, Aβ42/*p*-tau < 8.10 and Aβ42/Aβ40 < 0.069 (Lewczuk et al., 2018; Alcolea et al., 2019).

Statistical Analysis

Shapiro-Wilk test was used to investigate the distribution normality of the different variables. Demographic and clinical characteristics among diagnostic groups were compared using ANOVA or Kruskal–Wallis test for continuous variables, and Chi-square test (χ^2) or Fisher's exact test for categorical variables. Differences in BPSD and syndromic clusters' distribution among diagnostic groups were assessed with the Kruskal-Wallis test. Bonferroni correction was used to control for multiple comparisons in *post hoc* analyses performed through Dunn tests. Correlations between NPI scores and CSF biomarkers or visual atrophy brain scales were calculated using the Pearson coefficient. For non-parametric variables, confirmation was obtained using the Spearman coefficient. Differences in atrophy scores' distribution between patients with or without any of BPSD (e.g., agitation vs. non-agitation) were assessed with Fisher's exact tests. A beta regression model with backward elimination was used to evaluate the relations of the dependent variable, NPI total score, with the following baseline predictors: age, gender, education, MMSE, diagnosis, *t*-tau, Aβ42, left and right MTA, left and right PA, left and right GCA-F. The variable *p*-tau was removed after a first correlation analysis between the predictors. After the normalization of the dependent variable, the beta distribution was tested through a Kolmogorov-Smirnov test. Statistical computations were performed using R v. 3.5.3 (The R Foundation for Statistical Computing). Two-sided *p*-values <0.05 were considered to indicate significance. Due to the heterogeneous nature of the sample, *p*-values between 0.05 and 0.1 were also reported suggesting possible significance in further studies.

RESULTS

Demographic and clinical characteristics and biomarker measurements of the study population are shown in **Table 1**. Seventy-four patients were diagnosed with dementia (mean age, 74.5 ± 7.0 years; 60% female) and 26 with MCI (72.7 ± 6.5 years; 46%). The mean MMSE score was 17.3 ± 5.2 (range: 4.9–26.1) in patients with dementia and 26.8 ± 2.0 (range: 23–30) in subjects with MCI. In the dementia group, 48 patients were diagnosed as AD, seven as FTD, four as LBD, nine as VD, and six as Dem NOS. No significant difference was found among AD, non-AD, and MCI groups concerning age, gender, and education. As expected, the AD group showed lower Aβ42 levels, and higher *p*-tau and *t*-tau levels, compared to non-AD and MCI groups. No significant difference was found among the three groups with regard to atrophy scales scores.

Neuropsychiatric Symptoms Among Diagnostic Groups

NPI total score was not significantly different among diagnostic groups. Concerning the sub-items, delusions, hallucinations, and apathy were differently distributed among the diagnostic

TABLE 1 | Sociodemographic features, cognitive status, and biomarker measures in the total sample and in three main diagnostic groups.

	Total sample (*N* = 100)	Dementia (*N* = 74)		MCI (*N* = 26)	*p*-value[a]
		AD (*N* = 48)	non-AD (*N* = 26)		
Age, mean (sd), years	74.1 (6.9)	74.4 (6.5)	74.8 (8.0)	72.7 (6.5)	0.31
Female, *N* (%)	44 (44)	30 (62.5)	14 (53.8)	12 (46.2)	0.45
Education, mean (sd), years	7.8 (3.6)	7.5 (3.8)	7.8 (4.1)	8.2 (2.6)	0.43
MMSE, mean (sd)[b]	19.9 (6.2)	16.7 (5.4)	18.6 (4.7)	26.8 (2.0)	<0.001
NPI tot, mean (sd)	32.6 (30.0)	33.8 (31.8)	38.0 (29.3)	25.0 (26.7)	0.33
CSF biomarkers, mean (sd), pg/ml					
$A\beta_{42}$[b]	707.9 (360.2)	528.9 (230.9)	904.8 (394.0)	838.7 (376.5)	<0.001
p-tau[b]	72.5 (39.4)	95.7 (39.9)	44.6 (15.7)	57.4 (29.5)	<0.001
t-tau[b]	541.3 (351.8)	761.3 (360.3)	306.5 (117.2)	375.0 (235.3)	<0.001
MTA, N	91	44	23	24	
0 L/R	13/16	6/6	2/3	5/7	0.07/0.30
1 "	16/24	7/11	3/4	6/9	
2 "	36/28	16/15	8/7	12/6	
3 "	22/19	14/10	7/7	1/2	
4 "	4/4	1/2	3/2	0/0	
GCA, N	75	35	17	23	
0 L/R	18/18	7/7	2/2	9/9	0.24/0.30
1 "	27/28	13/14	6/6	8/8	
2 "	24/26	12/11	6/9	6/6	
3 "	6/3	3/3	3/0	0/0	
PA, N	76	36	17	23	
0 L/R	20/25	5/7	5/6	10/12	0.05/0.09
1 "	31/36	13/18	9/8	9/10	
2 "	21/11	14/7	3/3	4/1	
3 "	4/4	4/4	0/0	0/0	

AD, Alzheimer's disease; CSF, cerebrospinal fluid; GCA, global cortical atrophy; L, Left; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MTA, medial temporal atrophy; NPI, Neuropsychiatric Inventory; PA, posterior atrophy; R, right. [a]*Significance tests used were Fisher test for categorical variables and Kruskal–Wallis test for continuous variables.* [b]*Post hoc pair-wise comparisons with Bonferroni correction of diagnostic groups: MMSE: MCI > AD and non-AD ($p < 0.001$); $A\beta_{1-42}$: AD < non-AD and MCI ($p < 0.001$); p-tau: AD > non-AD and MCI ($p < 0.001$); t-tau: AD > non-AD and MCI ($p < 0.001$).*

groups ($p < 0.05$, <0.001, and <0.01, respectively), as well as the psychosis cluster when NPI clusters were computed ($p < 0.05$; **Figure 1**). After *post hoc* analyses, hallucinations showed higher scores in LBD compared to MCI and AD patients ($p < 0.001$ and $p < 0.05$, respectively), and the psychosis cluster also displayed higher scores in LBD concerning MCI subjects ($p < 0.05$).

Neuropsychiatric Symptoms and *t*-Tau

When searching for correlations between NPI and *t*-tau levels in patients with primary dementia (AD, LBD, and FTD), we found a trend toward a negative correlation ($R = -0.25$, $p = 0.08$; **Figure 2**). Moreover, after backward elimination, the beta regression model indicated a significant association between NPI total score and *t*-tau ($p < 0.01$; **Table 2**). As far as each sub-item of NPI was concerned, a significantly negative correlation was found between nighttime disturbances and *t*-tau both in patients with primary dementia ($R = -0.29$, $p < 0.05$) and in the total sample ($R = -0.22$, $p < 0.05$).

Figure 3 illustrates the relationship between *t*-tau and NPI total score according to the stage of disease (scored with MMSE). A positive non-significant relationship was observed in MCI

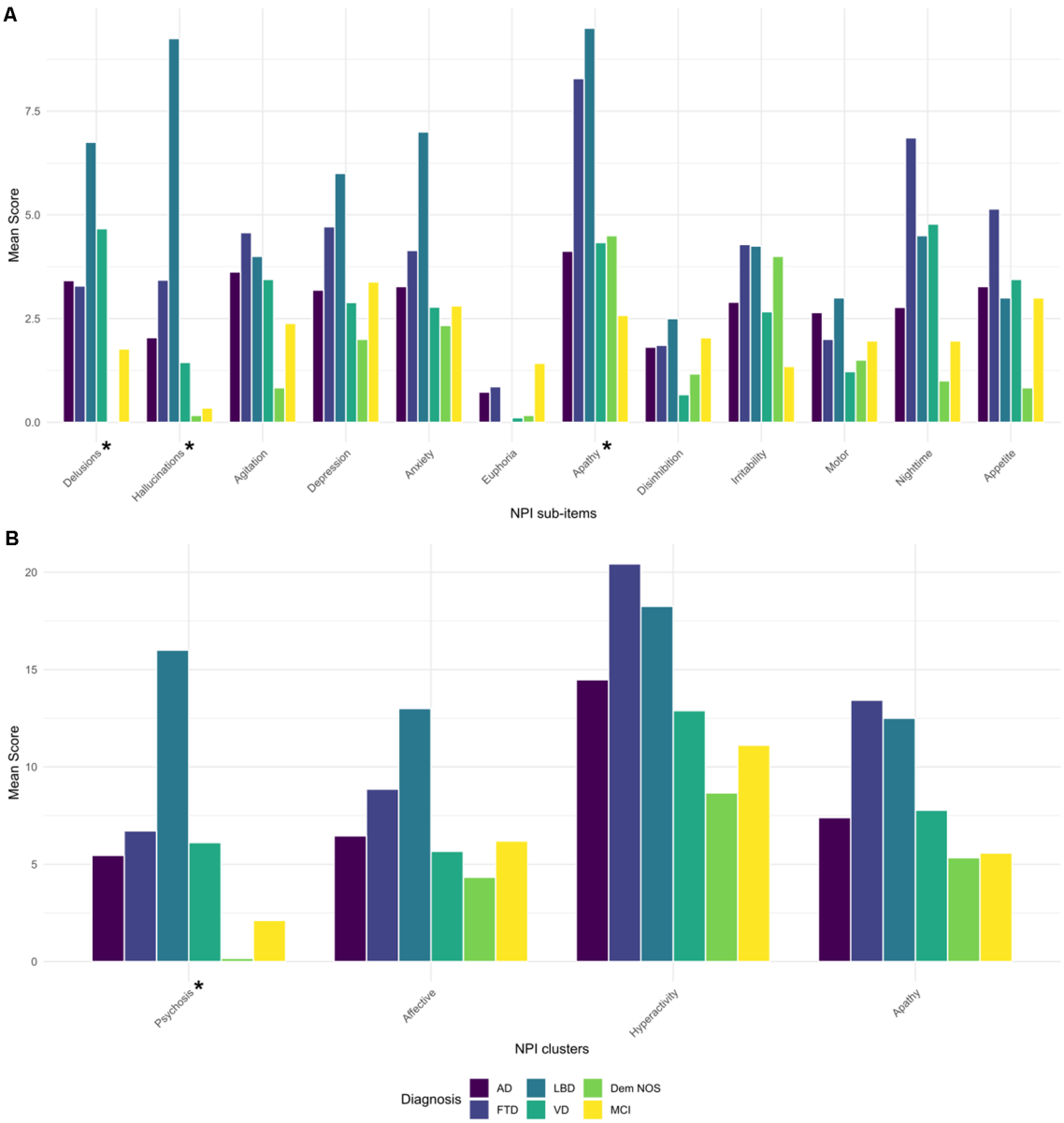

FIGURE 1 | Barplot of mean scores of NPI sub-items **(A)** and NPI clusters **(B)** by diagnostic groups. AD, Alzheimer's disease; FTD, frontotemporal dementia; Dem NOS, demented not otherwise specified; LBD, Lewy body dementia; MCI, mild cognitive impairment; NPI, Neuropsychiatric Inventory; VD, Vascular dementia. *Significance tests used were Kruskal–Wallis: Delusions ($p < 0.05$), Hallucinations ($p < 0.001$), Apathy ($p < 0.01$), Psychosis cluster ($p < 0.05$). *Post hoc* pair-wise comparisons with Bonferroni correction: Hallucinations: LBD > MCI and AD ($p < 0.001$ and 0.05, respectively), Psychosis cluster: LBD > MCI ($p < 0.05$).

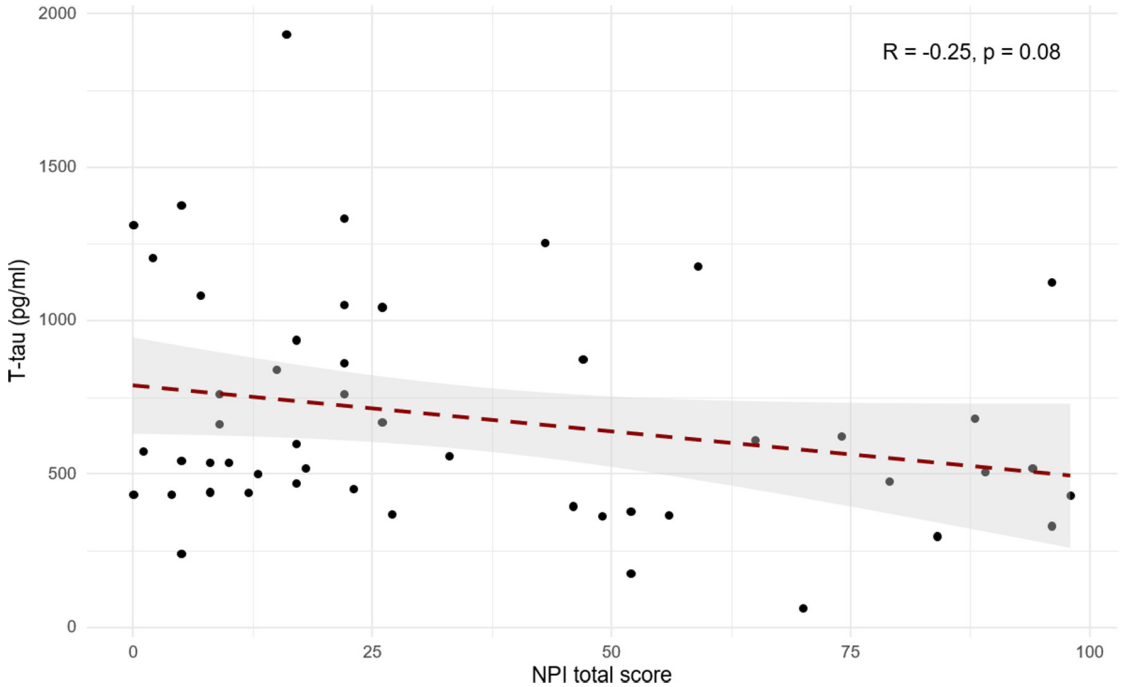

FIGURE 2 | Scatterplot of Pearson's correlation between cerebrospinal fluid (CSF) *t*-tau and NPI total score in patients with primary dementia (AD, FTD, and LBD).

TABLE 2 | Beta regression model of association of Neuropsychiatric Inventory (NPI) total score and sociodemographic and biomarker variables, displaying predictors retained in the final model after backward elimination.

	Estimate	Std. error	*Z*-value	*p*-value = pr(>|z|)
(Intercept)	4.204	2.144	1.961	0.050
Age	−0.053	0.026	−2.018	**0.044**
Male gender	−1.661	0.444	−3.741	**0.0002**
Education	−0.099	0.051	−1.932	0.053
MMSE	0.036	0.030	1.198	0.231
t-tau	−0.002	0.001	−2.855	**0.004**
$A\beta_{42}$	−0.001	0.001	−1.820	0.069
GCA L	−1.178	0.831	−1.418	0.156
GCA R	1.844	0.912	2.022	**0.043**

GCA, global cortical atrophy; L, Left; MMSE, Mini-Mental State Examination; R, right.

subjects, while a negative relationship was found in patients with dementia. Although the relationship is stronger in lower MMSE score, it shows a greater slope for higher MMSE score (MMSE = 12–23: $R = -0.07$, $p = 0.657$; MMSE = 0–11: $R = -0.55$, $p = 0.083$). The heterogeneity of the diagnostic groups included in the sample could partly explain the lack of significance observed in the correlations between *t*-tau and NPI total score (both in the whole sample and in the subgroups by MMSE range).

Neuropsychiatric Symptoms and Brain Atrophy

When patients were dichotomized into two groups based on the presence or absence of a specific NPI domain (e.g., agitation positive vs. agitation negative), significantly higher GCA-F scores were found in patients with the following symptoms (**Figure 4**): delusions ($p < 0.05$, on both sides), hallucinations ($p < 0.01$ and $p < 0.05$, on left and right side, respectively) and apathy ($p < 0.05$, on both sides). No significant differences were found regarding MTA and PA scores.

From correlation analyses, a positive correlation emerged between GCA-F scores and delusions (right: $p < 0.05$), agitation/aggression (left: $p < 0.05$), and psychosis cluster (right: $p < 0.05$). Conversely, nighttime disturbances were positively correlated with both GCA-F and MTA scores on both sides (left: $p < 0.01$; right: $p < 0.05$). Finally, following the correlation analyses, the beta regression model indicated a significant association between NPI total score and right GCA-F score ($p < 0.05$; **Table 2**).

DISCUSSION

This study investigated the distribution of neuropsychiatric symptoms and their CSF and neuroimaging correlate in a

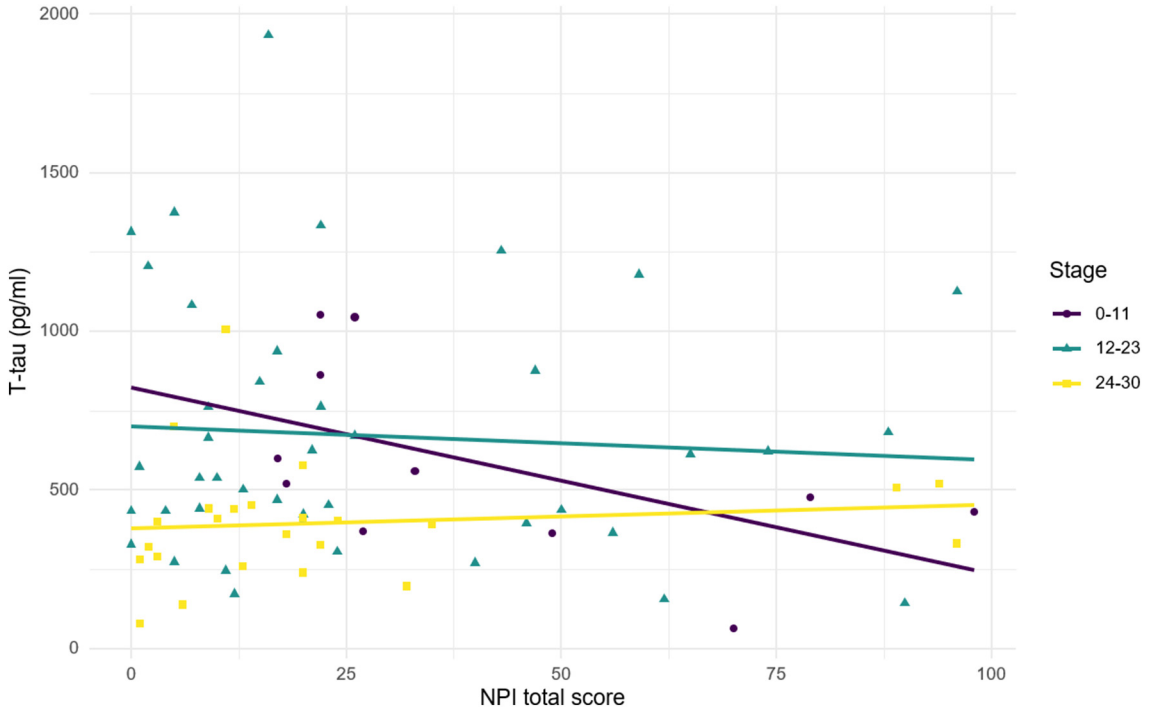

FIGURE 3 | Relationship between CSF *t*-tau and NPI total score according to disease stages. Stage was defined according to MMSE score. 24–30, mild cognitive impairment; 12–23, mild-to-moderate dementia; 0–11, moderate-to-severe dementia. Correlations: stage 24–30: $R = 0.11$, $p = 0.597$; stage 12–23: $R = -0.07$, $p = 0.657$; stage 0–11: $R = -0.55$, $p = 0.083$.

large sample of patients suffering from different dementing disorders, diagnosed with the most recent criteria. A significant prevalence of psychotic symptoms (hallucinations and delusions) was found in patients with LBD when compared to MCI or AD. The CSF biomarkers analysis showed a negative trend of *t*-tau when plotted with total NPI scores in patients with primary dementias, and a positive relationship in patients with MCI, drawing a parabolic trajectory across disease stages of increasing severity. However, the pathological heterogeneity of the sample and the small number of some etiological subgroups may have prevented the statistical significance of some analyses of correlation between *t*-tau and NPI score. Cortical atrophy in the frontal lobe was associated with the occurrence of delusions, hallucinations, and apathy, and correlated with the presence of agitation/aggression, while the atrophy of the medial temporal regions mainly correlated with night-time disturbances.

The high prevalence of hallucinations in our cohort of LBD patients is consistent with the most recent diagnostic criteria, which consider the hallucinations as one of the core clinical features, occurring in up to 80% of patients (McKeith et al., 2017) and predicting post-mortem Lewy pathology with 93% accuracy (Williams and Lees, 2005). This key symptom usually occurs in the form of well-structured visual hallucinations (VH), associated with different degrees of insight, even though tactile and auditory variants have been also reported (McKeith et al., 2017). Delusions are frequently reported in LBD, approximately in 49% of cases (Jellinger, 2012), the most frequent of them being represented by misidentification delusions, whose prevalence in LBD is higher than in AD (52% vs. 34%; Perini et al., 2016). The high frequency of psychotic symptoms observed in this study in LBD patients is therefore consistent with previous reports in the literature, supporting a higher prevalence of hallucinations and delusions in synucleinopathies, among which LBD, than in tauopathies, such as AD and FTD (Burghaus et al., 2012). Different functional and structural abnormalities have been suggested to underlie psychotic manifestations: parietal and occipital hypometabolism and frontal atrophy were reported in VH (Whitewell et al., 2007; Sanchez-Castaneda et al., 2010; Jellinger, 2012; Pezzoli et al., 2019), while hypoperfusion of frontal, limbic and paralimbic cortex was associated with misidentifications and delusions (Pernezckzy et al., 2008; Nagahama et al., 2010).

Interestingly, in our study, the negative trend observed between NPI total score and *t*-tau in patients with dementia was somewhat unexpected in front of the results of previous studies, which mostly report a positive correlation between *t*-tau and BPSD severity (Skogseth et al., 2008; Ramakers et al., 2013; Bloniecki et al., 2014; Babulal et al., 2016). Upon closer examination, these studies investigated populations with milder cognitive impairment than ours, including either healthy subjects (Babulal et al., 2016) or patients with MCI (Ramakers et al., 2013) or mild AD (MMSE = 24.2 ± 2.3; Skogseth et al., 2008).

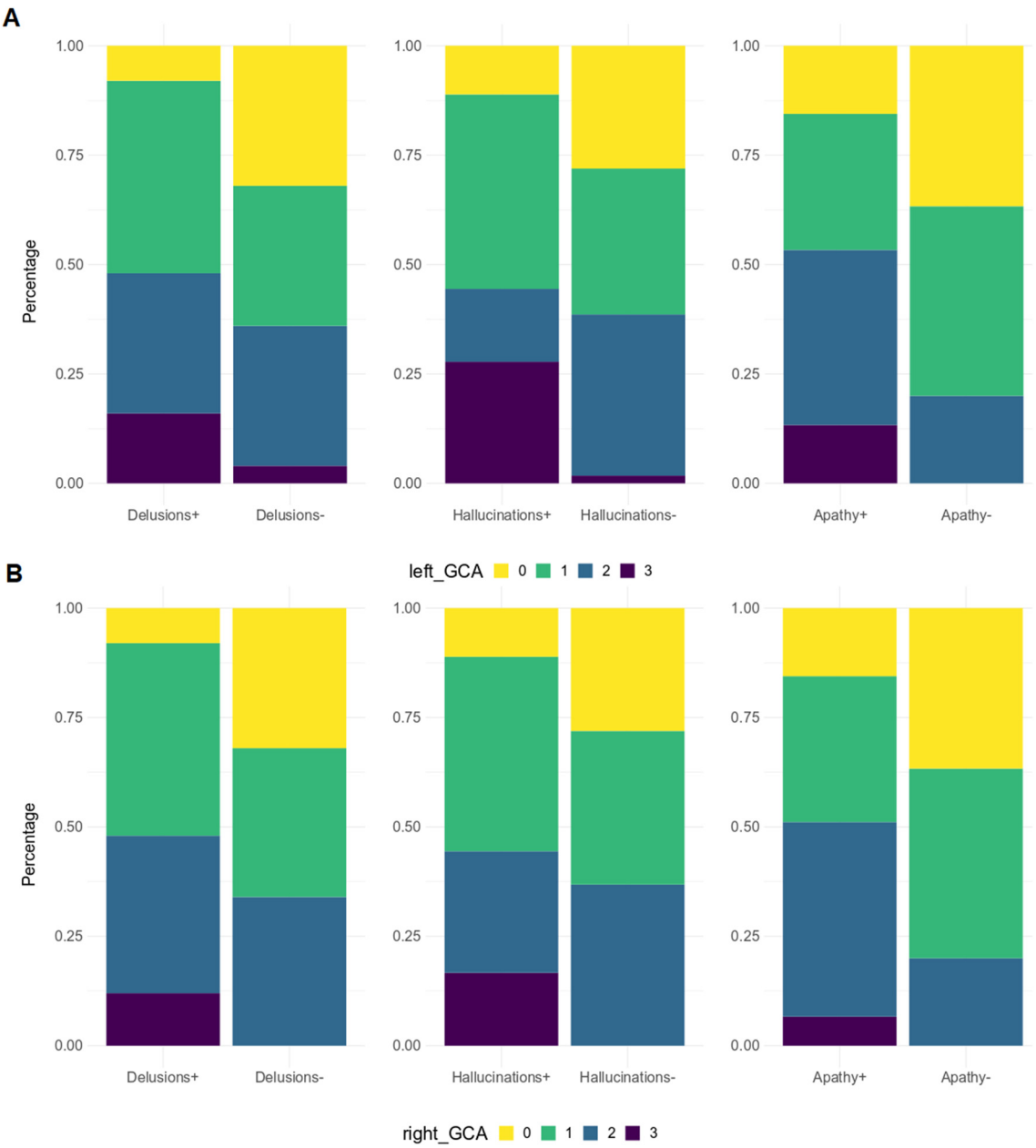

FIGURE 4 | GCA-F score distribution by NPI sub-items. GCA-F, global cortical atrophy; NPI, Neuropsychiatric Inventory. GCA-F scores were reported only for NPI sub-items with significant comparative changes [panel **(A)** for the left hemisphere, and panel **(B)** for the right hemisphere]. Significance tests used was Fisher test: Delusions ($p < 0.05$), Hallucinations ($p < 0.01$), Apathy ($p < 0.05$).

Conversely, the present study includes patients belonging to more advanced stages of the disease and with a more severe cognitive impairment, regardless of the etiological diagnosis (AD or non-AD; MMSE = 16.7 ± 5.4 and 18.6 ± 4.7, respectively). We, therefore, believe that *t*-tau decreases in advanced stages of dementia as a result of the decline of the neurodegenerative process involving the brain cortex, as also supported by longitudinal studies (Toledo et al., 2013). Unlike the above studies, Bloniecki and colleagues (Bloniecki et al., 2014) investigated the relationship between CSF biomarkers and BPSD in a cohort of cognitively more impaired patients (affected from mild-to-moderate AD, MMSE = 19.1 ± 4.2), and including a wider range of diagnosis (in addition to AD, also VD and AD mixed, MMSE = 20.2 ± 4.6). They found a positive correlation in the AD group alone, but with a decrease in the correlation coefficient (from 0.35 to 0.13) when the whole sample was analyzed (Bloniecki et al., 2014). The inclusion of non-AD dementias, usually characterized by lower *t*-tau values, could plausibly account for this finding. Similarly, the negative trend observed in our population of demented

patients could partly originate from the presence of non-AD dementias (in particular, FTD and LBD), which may contribute to attenuating or reversing the correlation between *t*-tau and BPSD. On the other hand, the finding of a positive trend in our subjects with MCI appears to be consistent with the evidence present in the literature related to early disease stages. To summarize, after an initial increase directly related to the spread of the neurodegenerative process (Fagan et al., 2009), CSF *t*-tau levels may decline in advanced disease stages, as a result of the reduced number of neurons spared by atrophy and still likely to degenerate. Consistently with this interpretation, Mollenhauer et al. (2005) reported a decline in *t*-tau levels in AD patients in the advanced stage, while Isoe et al. (1996) described a biphasic curve with an increase in *t*-tau levels at the disease onset, followed by a progressive decline in the final stages.

In this study, we report for the first time that cortex visual rating can detect more severe atrophy involving the frontal lobe in patients with delusions, hallucinations, or apathy. The search for possible links between BPSD and cortical abnormalities is currently an active field of investigation. A recent study identified the reduction of volume of the frontal lobe (in particular, the anterior cingulate cortex and the middle frontal gyrus) as a significant predictor of the occurrence of BPSD, such as apathy, delusions, and hallucinations, in AD patients (Boublay et al., 2020). Previously, other studies had supported this link, reporting a positive correlation between frontal atrophy and the occurrence of apathy in AD patients (Apostolova et al., 2007; Bruen et al., 2008), and an association between frontal lobe dysfunction and decline in the initiative in AD, FTD and LBD patients, particularly in late disease stages (Robert et al., 2006; Peavy et al., 2013; Massimo et al., 2015). The involvement of frontal networks subserving motivation and reward mechanisms, including the anterior cingulate cortex, superior and middle frontal gyri, and basal ganglia, provides a possible explanation of how atrophy involving these areas may contribute to the loss of interest and the development of apathy (Levy and Dubois, 2006; Moretti and Signori, 2016). Several studies also reported a more frequent finding of frontal atrophy in demented patients with hallucinations. In these subjects, Sanchez-Castaneda et al. (2010) precisely described a more severe cortical atrophy involving the inferior frontal gyrus and the precuneus, while Heitz et al. (2015) reported functional impairment of both anterior and posterior cortical regions, including the anterior cingulate cortex, the orbitofrontal cortex, and the cuneus. In addition to other reports in the literature (Whitewell et al., 2007; Pezzoli et al., 2019), this evidence appears to be endorsed by the results of the present study. The above frontal areas are indeed included in neuronal circuits assigned to inhibitory control and the decision-making mechanisms; their deregulation could prevent the patient from inhibiting the production of internal images, thus representing the pathophysiological substrate for the development of hallucinations (Shine et al., 2011). Conversely, understanding the neurobiological bases of delusions is more challenging, as few studies addressed systematically this issue. As mentioned above, frontal atrophy is a frequently encountered finding, that suggests the involvement of many areas of the frontal lobe in the generation of delusions, with particular regard to the orbitofrontal, limbic, and paralimbic regions (Mentis et al., 1995; Geroldi et al., 2000; Mega et al., 2000; Sultzer et al., 2003; Bruen et al., 2008; Pernezckzy et al., 2008; Nagahama et al., 2010). Also, the positive correlation between delusions' severity and right-sided GCA-F scores in our sample, confirms the suggested pivotal role of the right frontal lobe in controlling and structuring thought (Sultzer et al., 2003; Nakano et al., 2006; Devinsky, 2009). Based on this assumption, frontal cortical atrophy could promote the development of delusions through the loss of control functions aimed to supervise reality and to compare the internal experience with the outer world, leading to the consolidation of false beliefs (Richardson and Mallory, 1994). However, the meaning to attribute to the above findings is still largely speculative, mainly due to the heterogeneity of delusions' presentation, which may reflect the impairment of multiple functional networks located in different areas of the brain.

Further links emerged from correlation analyses between cortical atrophy and BPSD: agitation positively correlated with left-sided GCA-F scores, whereas night-time disturbances positively correlated with GCA-F and MTA scores on both hemispheres. The few existing studies of neuroimaging investigating structural and functional correlates of agitation/aggression reported an involvement of the left frontotemporal region in AD patients, associated with a concurrent more severe burden of neurofibrillary tangles in the same region (Tekin et al., 2001; Trzepacz et al., 2013). These findings are consistent with a large body of neuropsychiatry literature describing a complex brain network of prefrontal, subcortical, and mesolimbic circuitry that mediates and regulates social behaviors, and of frontoinsular circuitry that plays a crucial role in the processing of more complex social emotions such as empathy, compassion, and fairness (Menon and Uddin, 2010). Agitation and aggression may therefore be due to the default of this frontotemporal network, leading to the loss of capacity to process and regulate behaviors properly (Trzepacz et al., 2013).

Sleep disorders are a particularly disabling aspect common to many forms of dementia, representing stressful conditions for patients and caregivers, and a major risk factor for early institutionalization. Micro-architectural sleep alterations, nocturnal sleep fragmentation, decrease in nocturnal sleep duration, diurnal napping, and even inversion of the sleep-wake cycle are the main disorders observed in patients with AD (Peter-Derex et al., 2015). In the present study, a positive correlation was found between night-time disorders and medial temporal atrophy; nonetheless, other structures involved in arousal regulation, as the hypothalamic suprachiasmatic nucleus (SCN), was previously correlated to sleep disruption (Lyketsos et al., 2007). It was proposed that MTA may induce dysfunction of the SCN and/or its downstream effector systems, and that, in turn, the fragmented and irregular rhythms of activity may aggravate the neuropathological process responsible

for the MTA, according to a negative feed-forward cycle (Musiek et al., 2015; Van Someren et al., 2019).

LIMITATIONS

The main limitation of this study is the heterogeneous composition of the population and the small size of some subgroups. The larger number of AD patients may have partly affected the correlation analyses between CSF or neuroimaging biomarkers and BPSD severity. Indeed, primary dementia, such as AD, FTD, and LBD, usually show different levels of tau and different cortical atrophy distribution, as a result of the underlying neuropathological process and its specific tropism. Moreover, some kinds of dementia are known to be characterized by specific BPSD, and these are purposely included in the diagnostic criteria (e.g., apathy in FTD, and hallucinations in LBD). The variable contribution of these nosological entities to the overall study population may have affected the percent presentation of BPSD, and may therefore have introduced a selection bias in the analyses performed on the whole sample. Finally, the small size of FTD and LBD groups may have been a limiting factor influencing the significance in the analyses according to sub-groups.

CONCLUSIONS

This study provides a real-world overview of the most clinically relevant BPSD occurring in patients consecutively attending a memory clinic due to dementing conditions. The gathered evidence suggests that, in a future perspective, CSF biomarkers and visual rating scales for cortical atrophy could be hopefully included in a multidimensional evaluation of demented patients, aimed to predict prognosis and occurrence of BPSD. Longitudinal studies on wider and diagnosis-balanced cohorts of patients are however necessary to properly ascertain the actual predictive value of these biomarkers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MCR, GP, and AC: conceptualization. MCR, GP, MC, MP, DF, and LF: methodology; MCR, GP, BD, and EB: formal analysis and investigation. MCR, GP, and GV: writing—original draft preparation. MCR, GP, AC, DF, and EB: writing—review and editing. AC: resources and supervision. All authors contributed to the article and approved the submitted version.

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A Meta-Analysis of Brain DNA Methylation Across Sex, Age, and Alzheimer's Disease Points for Accelerated Epigenetic Aging in Neurodegeneration

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Alzheimer's disease (AD) is characterized by specific alterations of brain DNA methylation (DNAm) patterns. Age and sex, two major risk factors for AD, are also known to largely affect the epigenetic profiles in brain, but their contribution to AD-associated DNAm changes has been poorly investigated. In this study we considered publicly available DNAm datasets of four brain regions (temporal, frontal, entorhinal cortex, and cerebellum) from healthy adult subjects and AD patients, and performed a meta-analysis to identify sex-, age-, and AD-associated epigenetic profiles. In one of these datasets it was also possible to distinguish 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) profiles. We showed that DNAm differences between males and females tend to be shared between the four brain regions, while aging differently affects cortical regions compared to cerebellum. We found that the proportion of sex-dependent probes whose methylation is modified also during aging is higher than expected, but that differences between males and females tend to be maintained, with only a few probes showing age-by-sex interaction. We did not find significant overlaps between AD- and sex-associated probes, nor disease-by-sex interaction effects. On the contrary, we found that AD-related epigenetic modifications are significantly enriched in probes whose DNAm varies with age and that there is a high concordance between the direction of changes (hyper or hypo-methylation) in aging and AD, supporting accelerated epigenetic aging in the disease. In summary, our results suggest that age-associated DNAm patterns concur to the epigenetic deregulation observed in AD, providing new insights on how advanced age enables neurodegeneration.

Keywords: DNA methylation, Alzheimer's disease, brain, sex, aging

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disease that leads to a progressive decay of cognitive abilities and self-sufficiency. Neuronal loss involves multiple brain regions that are progressively affected by the disease. Hippocampus and entorhinal cortex exhibit the earliest pathological changes, preceding the onset of clinical signs and cognitive impairment by several years, and later the disease spreads to the other brain regions (Braak and Braak, 1991; Van Hoesen et al., 1991; Scahill et al., 2002; Coupé et al., 2019).

Advanced age and female sex are the two major non-modifiable risk factors for AD (Hickman et al., 2016; Podcasy and Epperson, 2016; Fisher et al., 2018). More than 95% of cases of AD occur after 65 years of age (late onset AD), and AD prevalence increases exponentially between 65 and 85 years (Hebert et al., 1995; Kawas and Corrada, 2006). Two-thirds of clinically diagnosed cases of AD are women, and the fact that women live longer than man does not fully explain this sex bias for AD (Pike, 2017; Nebel et al., 2018).

The etiology and pathogenesis of AD are complex and likely result from the interplay between genetic and environmental factors during lifespan. In this scenario epigenetic modifications have attracted increased interest in the study of AD, as they integrate genetic background and environment and modulate genomic organization and gene expression. Epigenetic modifications regulate brain biology throughout development and lifetime, influencing neuronal plasticity, cognition, and behavior (Fagiolini et al., 2009), and deregulation of brain epigenetic patterns has been associated to the pathogenesis of neurological and psychiatric disorders (Landgrave-Gómez et al., 2015; Jaffe et al., 2016). Several studies in post-mortem AD brains have investigated the role of DNA methylation (DNAm), the best-characterized epigenetic modification, identifying a number of CpG sites that show robust changes in DNAm compared to non-demented controls (Lunnon et al., 2014; Gasparoni et al., 2018; Smith et al., 2018, 2019, 2020; Altuna et al., 2019; Lardenoije et al., 2019; Semick et al., 2019; Smith R. G. et al., 2020; Wei et al., 2020).

Interestingly, the two major non-modifiable AD risk factors mentioned above, i.e., sex and age, are also among the main biological variables that influence epigenetic patterns in most human tissues, including brain (Gilbert et al., 2019).

Genome-wide DNAm differences between males and females have been found in whole blood (Singmann et al., 2015) and have been related to the sex-biased risk of psychiatric diseases (Maschietto et al., 2017). A similar link has been reported also in brain (Xia et al., 2019) where sex-specific DNAm patterns are established early during prenatal development (Spiers et al., 2015; Perzel Mandell et al., 2020) and are at least in part maintained in the adulthood (Xu et al., 2014; Spiers et al., 2015), contributing to the profound differences in brain functions between males and females (McCarthy et al., 2009; Forger, 2016; Gegenhuber and Tollkuhn, 2019) and to the different onset of psychiatric disorders (Perzel Mandell et al., 2020).

DNAm patterns are largely remodeled during aging (Pal and Tyler, 2016), where a trend toward global loss of DNAm together with hypermethylation at specific loci is observed (Xiao et al., 2019). Although with some differences among brain regions (Hernandez et al., 2011; Horvath et al., 2015), age-associated epigenetic changes interest also the brain, likely contributing to the structural and functional alterations that can result in progressive cognitive decline and increased susceptibility to neurodegenerative disorders (Bishop et al., 2010; Lardenoije et al., 2015).

So far, only few studies have considered how sex and age interact during lifespan in shaping the epigenome. Data on whole blood indicate that sex-dependent DNAm is remodeled during aging (McCartney et al., 2019), and we suggested that these changes occur at different extent in human models of successful and unsuccessful aging (Yusipov et al., 2020). In mouse hippocampus and human frontal cortex, Masser et al. identified both CpGs in which sex-dependent DNAm is maintained during lifetime, and CpG sites that are differentially affected by aging in relation to sex (Masser et al., 2017). Interestingly, some studies employing epigenetic clocks, i.e., DNAm-based predictors of age, reported accelerated aging in whole blood from males compared to females (Horvath et al., 2016; Xiao et al., 2018; Tajuddin et al., 2019), and the same trend was observed also in brain (Horvath et al., 2016).

Collectively, the available data sustain the importance of sex and aging in shaping the brain epigenome, but so far only one study combined different datasets to identify reproducible sex-associated DNAm profiles (Xia et al., 2019). No study has systematically analyzed multiple datasets and brain regions to identify DNAm patterns resulting from the interaction of sex and age during lifespan, and most importantly no study has evaluated whether sex- and age-dependent DNAm can contribute to epigenetic deregulation in AD, despite the pivotal role of these two factors in AD etiology and pathogenesis.

To fill this gap, in the present paper we performed a meta-analysis of DNAm across sex, age, and AD considering publicly available datasets from different brain regions.

Abbreviations: DNAm, DNA methylation; AD, Alzheimer's disease; DMPs, differentially methylated positions; EWAS, epigenome-wide association study; GO, gene ontology; sDMPs, sex-associated differentially methylated positions; aDMPs, age-associated differentially methylated positions; s&aDMPs, sex-, and age-associated differentially methylated positions; AD&aDMPs, late onset Alzheimer's disease-specific age-associated differentially methylated positions; AD&sDMPs, late onset Alzheimer's disease-specific sex-associated variably methylated positions; AD&a&sDMPs, late onset Alzheimer's disease-specific sex- and age-associated variably methylated positions; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5uC, unmethylated cytosine; BS, bisulfite; oxBS, oxidative bisulfite.

MATERIALS AND METHODS

Datasets

To select DNAm datasets based on Infinium BeadChip technology, the Gene Expression Omnibus (GEO) repository (Clough and Barrett, 2016) was interrogated by the *GEOmetadb* Bioconductor package using the following search terms: "GPL13534," "GPL21145," to include only datasets based on the Illumina Infinium HumanMethylation450 and MethylationEPIC BeadChips; "sex," "gender," "female," to include only datasets in

which the information on the sex of the subjects was available; "age," to include only datasets in which the information on the age of the subjects was available; "brain," "cortex," "gyrus," "lobe," "gray," to select datasets in which brain samples were analyzed; "control," "normal," "non-tumor," "health," or "Alzheimer," "AD," "Braak," to select datasets including healthy and AD subjects, respectively. We considered only datasets including more than 10 healthy subjects. As to June 30th 2020, only Illumina Infinium HumanMethylation450 datasets were retrieved.

For the meta-analysis of sex- and age-dependent DNAm in healthy subjects, we selected only datasets including at least 10 males and 10 females, having more than 19 years and spanning an age range of at least 30 years. We further considered only brain regions for which at least two datasets were available. This resulted in eight datasets covering four regions: Frontal cortex (FC), Temporal cortex (TC), Entorhinal cortex (ERC), Cerebellum (CRB) (**Table 1**).

For the meta-analysis of AD-associated methylation patterns, we selected only the datasets including subjects over 65 years of age with at least 3 males and 3 females in the control and AD groups. This resulted in eight datasets covering the same brain regions indicated above (**Table 2**).

Pre-processing

As raw intensities files were not available for some datasets, all the analyses were performed on pre-processed methylation data downloaded from GEO. Potentially ambiguous probes (cross-reactive probes and probes including SNPs; Zhou et al., 2017) were excluded from the analyses. Probes mapping on sex chromosomes were removed, except when the comparison between AD and healthy controls was performed in males and females separately. GSE134379, GSE125895, GSE66351, and GSE76105 did not include probes mapping on sex chromosomes in the pre-processed data downloaded from GEO.

TABLE 1 | Characteristics of the Infinium450k datasets including healthy subjects selected in the present study for the meta-analysis of sex- and age-associated DNAm.

Number of GEO accession	Regions	Number of subjects	Sex (F/M)	Age range (years)
GSE105109	Entorhinal cortex	27	13/14	58–99
	Cerebellum	28	14/14	58–99
GSE125895	Frontal cortex	47	19/28	51.83–83.64
	Entorhinal cortex	49	20/29	51.83–83.64
GSE134379	Temporal cortex	179	76/103	63–103
	Cerebellum	179	76/103	63–103
GSE59685	Frontal cortex	24	12/12	55–95
	Temporal cortex	26	13/13	40–95
	Cerebellum	23	10/13	40–95
GSE74193	Frontal cortex	216	68/148	19.26–85.2
GSE64509	Frontal cortex	40	22/18	32–114
	Cerebellum	31	21/10	38–114
GSE66351	Frontal cortex	25	10/15	46–88
	Temporal cortex	25	10/15	46–88

In each dataset, neuron/glia proportions were estimated using Horvath's calculator (Horvath, 2013) which implements the algorithm developed by Guintivano et al. (2013).

For the analysis of 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), and unmethylated cytosine (5uC) in the GSE105109 dataset, we considered only the samples for which both bisulfite (BS) and oxidative bisulfite (oxBS) were available. ERC included 25 healthy subjects (12 females and 13 males) and 57 AD (25 females and 32 males), while CRB included 28 healthy subjects (14 females and 14 males) and 63 AD (26 females and 37 males). OxBS beta values correspond to 5mC levels; 5hmC levels were calculated by subtracting oxBS

TABLE 2 | Characteristics of the Infinium450k datasets investigated in the present study including AD patients and non-demented control subjects.

Number of GEO accession	Regions	Number of subjects	Sex (F/M)	Age range (years)
GSE105109	Entorhinal cortex Ctrl	24	13/11	66–99
	Entorhinal cortex AD	61	27/34	67–97
	Cerebellum Ctrl	25	13/12	66–99
	Cerebellum AD	64	27/37	67–97
GSE125895	Frontal cortex Ctrl	11	5/6	65.04–83.64
	Frontal cortex AD	18	9/9	71.47–92.29
	Entorhinal cortex Ctrl	12	5/7	65.04–83.64
	Entorhinal cortex AD	17	10/7	71.47–92.29
	Cerebellum Ctrl	8	4/4	65.04–83.64
	Cerebellum AD	20	11/8	71.47–92.29
GSE134379	Temporal cortex Ctrl	175	76/99	68–103
	Temporal cortex AD	217	117/100	66–102
	Cerebellum Ctrl	175	74/95	68–103
	Cerebellum AD	217	117/100	66–102
GSE59685	Frontal cortex Ctrl	21	10/11	66–95
	Frontal cortex AD	60	39/21	66–103
	Temporal cortex Ctrl	22	11/11	66–95
	Temporal cortex AD	61	40/21	66–103
	Entorhinal cortex Ctrl	19	8/11	66–95
	Entorhinal cortex AD	58	19/13	66–95
	Cerebellum Ctrl	19	8/11	66–95
	Cerebellum AD	60	39/21	66–103
GSE66351	Frontal cortex Ctrl	12	8/4	71–88
	Frontal cortex AD	35	22/13	67–97
	Temporal cortex Ctrl	12	8/4	71–88
	Temporal cortex AD	37	23/14	67–97
GSE76105	Temporal cortex Ctrl	34	18/16	66–94
	Temporal cortex AD	34	17/17	66–92
GSE80970	Frontal cortex Ctrl	68	34/34	70–108
	Frontal cortex AD	74	54/30	72–103
	Temporal cortex Ctrl	70	36/34	70–108
	Temporal cortex AD	74	54/30	72–103
GSE109627	Temporal cortex Ctrl	36	19/17	73–94
	Temporal cortex AD	46	24/22	70–95

Ctrl, healthy subjects; AD, Alzheimer's disease patients.

beta values from BS beta values (BS-oxBS), while 5uC levels were calculated by subtracting BS beta values from 1 (1-BS; Lardenoije et al., 2019). Negative values returning from the difference BS-oxBS were set to a value close to zero (1×10^{-7}; Ringh et al., 2019).

Differential Analysis and Meta-Analysis

To identify differentially methylated positions (DMPs), the *lmFit* function implemented in *limma* R package (Ritchie et al., 2015) was used to fit a linear model to each microarray probe, expressing DNAm as M-values. Association with age was calculated using age as a continuous value and correcting for sex and neuron/glia proportion. Association with sex was calculated using sex as a categorical value and correcting for age and neuron/glia proportion. Association with AD was calculated using AD as a categorical value and correcting for age, sex and neuron/glia proportion. The *lmFit* function was used also to calculate the interaction between sex and age, correcting for neuron/glia proportion, and between AD and sex, correcting for age and neuron/glia proportion. Effect sizes and standard errors were extracted from *limma* output. For each brain region, the results obtained in the different datasets were combined by inverse variance-weighted fixed-effects meta-analysis using METAL software (Willer et al., 2010). Finally, the *p*-values resulting from each meta-analysis were adjusted for multiple comparisons using the Benjamini-Hochberg (BH) procedure. Only probes with a BH-corrected *p*-value <0.01 and with concordant effect sizes between all the datasets included in each meta-analysis were retained as significant.

To identify DMPs specific for a certain brain region, we first selected the probes having a BH-corrected *p*-value <0.01 in one region and a BH-corrected *p*-value >0.01 in all the other regions; we further refined these lists by selecting the probes having large effect sizes (<5th percentile or >95th percentile) in the brain region under investigation and small absolute effect sizes (<0.1 for sex analysis; <0.001 for age analysis; <0.1 for AD analysis) in all the other regions.

Enrichment and Gene Ontology Analysis

Enrichment of genomic regions (islands, N- and S-shores and shelves, open sea regions) was calculated using Fisher exact test, as implemented in the *fisher.test* function from the *stats* R package (*p*-value <0.05). Enrichment of Gene Ontology (GO) terms was calculated using the *methylgometh* function implemented in the *methylGSA* R package (Ren and Kuan, 2019), and redundant significant GO terms (BH-corrected *p*-value <0.01) were removed by REViGO software (Supek et al., 2011).

RESULTS

The selection criteria of publicly available DNAm datasets of healthy and AD human brains are described in Materials and Methods section, and the datasets included in the meta-analysis are reported in **Tables 1**, **2**. An overview of the study design is reported in **Supplementary Figure 1**.

DNA Methylation Differences Across Sex

To identify sex-dependent differentially methylated positions (sDMPs) we performed an epigenome wide association study (EWAS) in each dataset and brain region separately, considering healthy subjects and correcting for age and estimated neuron/glia proportion (Section Materials and Methods). We then conducted a meta-analysis within each brain region.

We identified 4,860 sDMPs in FC, 1,985 sDMPs in TC, 159 sDMPs in ERC, and 2,322 sDMPs in CRB (**Figures 1A–D**, **Supplementary Figure 2**, and **Supplementary File 1**).

In FC, sDMPs were mainly hypermethylated in males compared to females (73% of hypermethylated probes) while the opposite was true for TC, ERC, and CRB (38, 33, and 36% of hypermethylated probes in TC, ERC, and CRB, respectively). When analyzing the genomic context of the sDMPs, we found that CpG islands were enriched in sDMPs in all the four brain regions, and that CpG island shores showed a similar trend (**Supplementary File 2**). Also the distribution of sDMPs across chromosomes was not random, with a trend toward enrichment in chromosome 19 in all the four brain regions. The enrichment analysis of GO terms did not reveal significant results except for FC, where the "homophilic cell adhesion via plasma membrane adhesion molecules" ontology was found (**Supplementary File 2**).

To investigate whether sex-dependent DNAm changes were consistent across brain regions, we evaluated the correlation of effect size values between FC, TC, ERC, and CRB (**Figure 2A**). The four brain regions were positively correlated each other. We next intersected the 4 sDMPs lists, identifying 77 common probes mapping in 57 genes (**Figure 2D**, **Table 3**, and **Supplementary File 1**).

All these probes showed concordant sex-dependent DNAm profiles in the four brain regions and most of them (73%) were hypomethylated in males. Furthermore, 93% of them were previously described to have sex-dependent DNAm also in whole blood (Yusipov et al., 2020).

On the other hand, we searched for probes having sex-related DNAm differences only in one brain region (region-specific sDMPs; Section Materials and Methods). We found 2, 4, 0, and 37 region-specific sDMPs in FC, TC, ERC, and CRB, respectively (**Supplementary File 1**). Interestingly five sDMPs specific for CRB mapped in Nuclear Enriched Abundant Transcript 1 (NEAT1) gene (**Figure 3**).

DNA Methylation Changes Across Age

To identify age-dependent differentially methylated positions (aDMPs) we performed an EWAS in each dataset and brain region separately, considering healthy subjects and correcting for sex and estimated neuron/glia proportion (Section Materials and Methods). We then conducted a meta-analysis within each brain region.

We identified 24,581, 10,077, 404, and 1,140 aDMPs in FC, TC, ERC, and CRB, respectively (**Figures 1E–H**, **Supplementary Figure 3E**, and **Supplementary File 3**). In all brain regions, most of the aDMPs underwent hypermethylation with age (76, 88, 58, and 62% of hypermethylated aDMPs

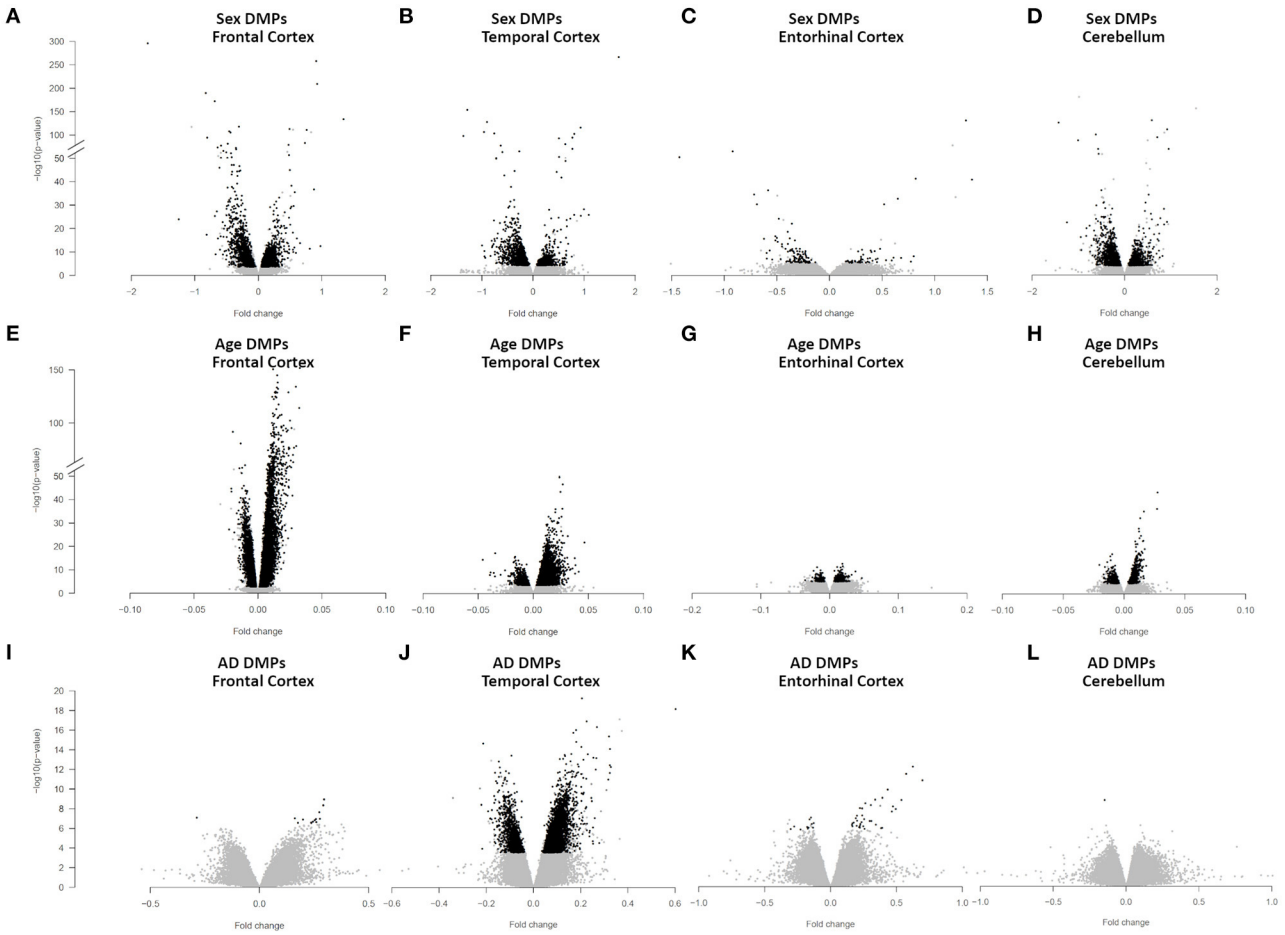

FIGURE 1 | Sex-, age-, and AD-associated epigenetic changes in the four brain regions. Volcano plots of -log10(*P*-value) against effect sizes, resulting from the meta-analysis of: (i) sex-associated DMPs in FC **(A)**, TC **(B)**, ERC **(C)**, and CRB **(D)**; (ii) age-associated DMPs in FC **(E)**, TC **(F)**, ERC **(G)**, and CRB **(H)**; (iii) AD-associated DMPs in FC **(I)**, TC **(J)**, ERC **(K)**, and CRB **(L)**. Significant probes (BH-corrected *p*-value <0.01) are colored in black.

in FC, TC, ERC, and CRB, respectively). The genomic context of aDMPs was not consistent across the four brain regions, except for a significant under-representation in "open sea" regions (**Supplementary File 4**). Similarly, aDMPs were differently scattered across chromosomes in FC, TC, ERC, and CRB. GO enrichment analysis revealed several pathways involved in morphogenesis and developmental processes, with "pattern specification process" and "regionalization" common to FC, TC, and ERC (**Supplementary File 4**).

The analysis of correlation between the effect sizes revealed that age-associated changes were more similar between FC and TC compared to the other regions (**Figure 2B**). The intersection of the aDMPs from the 4 brain regions highlighted 28 common probes, all concordantly undergoing hypermethylation with age and mapping in 25 genes (**Figure 2E** and **Table 4**). Again, 93% of these probes were reported as age-associated also in while blood (Yusipov et al., 2020).

The opposite analysis, i.e., the identification of region-specific aDMPs (section Materials and Methods), identified only one probe specific for FC (cg01725130), that maps in the body of Ras And Rab Interactor 3 (RIN3) gene (**Supplementary File 2**).

The Relation Between Age and Sex in Brain DNA Methylation

We then aimed at studying how sex-specific brain DNAm is modulated during aging.

First of all, we intersected sDMPs and aDMPs lists. In FC, we found 675 probes that change with sex and with age (s&aDMPs), corresponding to about 13% of all sDMPs identified. In TC s&aDMPs were 171, corresponding to 8.5% of sDMPs. In ERC we found only 2 s&aDMPs, while in CRB s&aDMPs were 19, corresponding to 4% of sDMPs (**Figure 4** and **Supplementary Files 1**, **3**). In all the four regions, the proportion of sDMPs changing with age (i.e., the proportion of s&aDMPs) was higher than expected (Fisher's Exact Test *p*-value <0.05; odds ratio of 2.6, 3.8, 13.0, and 3.0 in FC, TC, ERC, and CRB, respectively). In FC, TC, and CRB, most

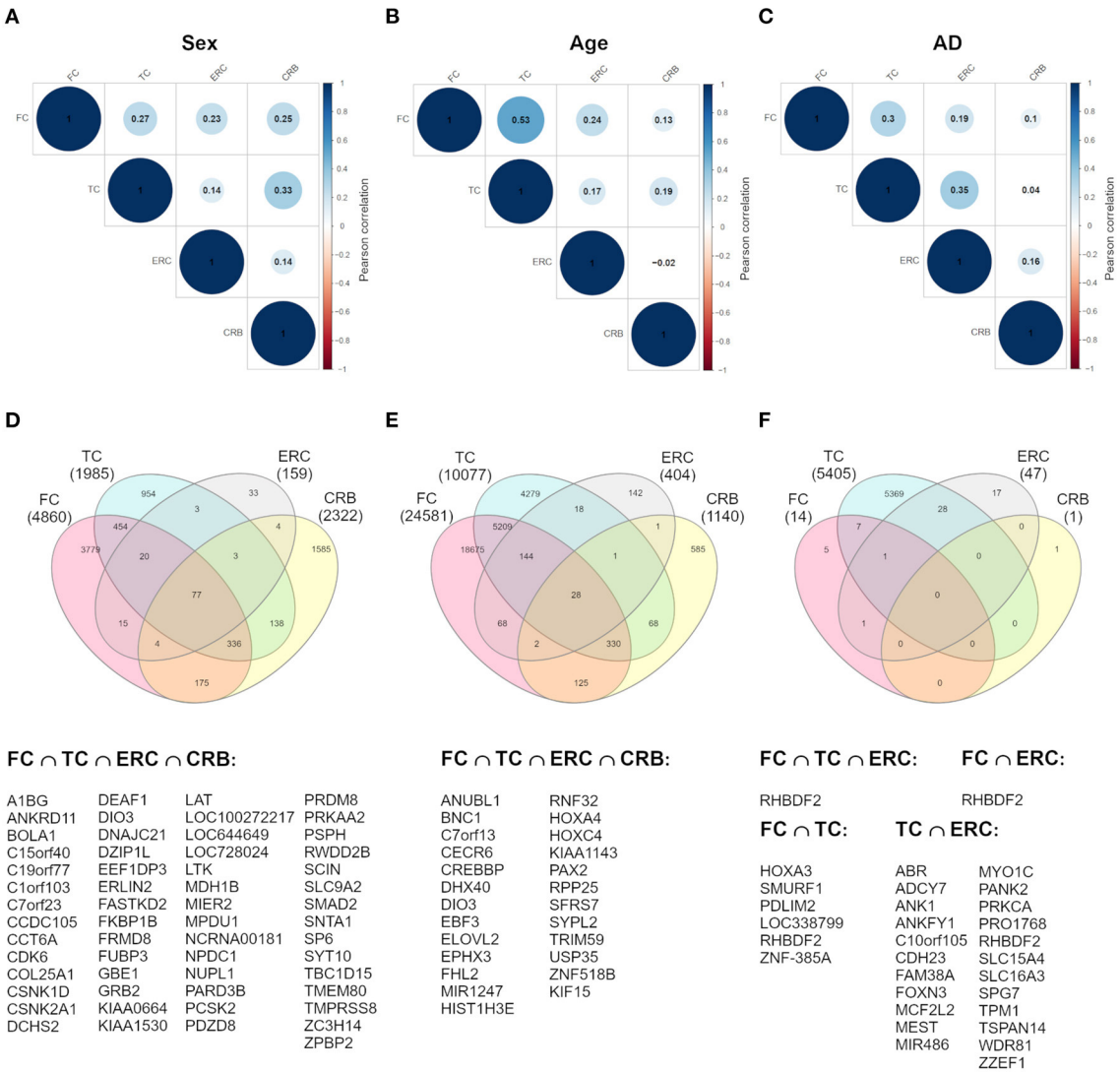

FIGURE 2 | Cross-region analysis of sex-, age-, and AD-associated probes. **(A–C)** The correlation matrix plots show the magnitude of correlation among probes' effect sizes in the four brain regions, considering the results of the meta-analysis on sex- **(A)**, age- **(B)**, and AD- **(C)** associated probes. Positive and negative correlation values are indicated in blue and red, respectively. **(D–F)** The Venn diagrams display the number of significant DMPs shared between the four brain regions, considering sDMPs **(D)**, aDMPs **(E)**, and AD-DMPs **(F)**. The genes in which the most shared probes map are reported below each diagram.

of the s&aDMPs were probes having higher DNAm levels in males respect to females and undergoing hypermethylation during aging. GO analysis revealed only one ontology enriched in FC ("homophilic cell adhesion via plasma membrane adhesion molecules").

The previous analysis identifies CpG probes whose DNAm varies according to both sex and age, but is not informative about possible differences in aging trajectories between males and females. To fulfill this point, we performed an age-by-sex interaction analysis in each dataset (Section Materials and Methods) and meta-analyzed the results for the four brain regions. Only 4, 4, 2, and 2 probes showed a significant age-by-sex interaction in FC, TC, ERC, and CRB, respectively (**Supplementary File 5**).

Brain DNA Methylation Changes Across AD

Then, we focused on brain DNAm datasets including late-onset AD patients and age-matched non-demented controls.

To identify differentially methylated positions associated with AD (AD-DMPs) we performed an EWAS in each dataset and brain region separately, correcting for age, sex, and estimated neuron/glia proportion (section Materials and Methods). We then conducted a meta-analysis within each brain region.

We identified 14 AD-DMPs in FC, 5405 in TC, 47 in ERC, and only 1 in CRB (**Figures 1I–L**, **Supplementary Figure 4**, and **Supplementary File 6**). In all brain regions most of AD-DMPs were hypermethylated in AD compared to controls (93, 80, 76, and 100% in FC, TC, ERC, and CRB, respectively). While in TC AD-DMPs were significantly under-represented

TABLE 3 | sDMPs resulting from cross-region analysis.

Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Yusipov et al.	Cited in previous studies in relation to sex
cg00097357	12	33591336	N_Shore	SYT10	-	X	
cg00655923	7	64895418			+	X	
cg00760935	4	15541	Island	DCHS2	-	X	
cg01063965	11	695461	Island	TMEM80, DEAF1	-		Involved in sex-dependent anxiety and depression (Luckhart et al., 2016; Philippe et al., 2018)
cg01181499	2	74739419	N_Shore		-	X	
cg01906879	3	81811016	S_Shore	GBE1	-	X	
cg02093808	4	77342011	Island		-	X	
cg02297043	1	75590912	Island		-	X	
cg02530860	8	14436	Island		+	X	
cg03168896	3	44036098	N_Shore		-	X	
cg03405128	4	77341841	N_Shore		-	X	
cg03687700	2	24271844	N_Shore	FKBP1B	-	X	
cg03894796	8	13783	Island		+	X	
cg04946709	16	59789030	Island	LOC644649	+	X	
cg05020125	8	37605552		LOC728024, ERLIN2	-	X	
cg05056638	8	24800824	S_Shore		-	X	
cg05100634	18	45457604	Island	SMAD2	-		Sex-differences in extracellular matrix production (Wu et al., 2015; Dworatzek et al., 2016; Altinbas et al., 2019; Avouac et al., 2020)
cg05468028	21	30391383	Island	RWDD2B	-	X	
cg05849319	11	65172370	Island	FRMD8	+	X	
cg06666376	19	3480596	N_Shore	C19orf77	+	X	
cg06710937	13	23489940	Island		-	X	
cg07462804	4	81105375	Island	PRDM8	-	X	
cg07645761	16	2892518	N_Shore	TMPRSS8	+	X	
cg07953307	16	29000920		LAT	+	X	
cg08541880	3	13783	Island	DZIP1L	-	X	
cg09045105	1	149871	Island	BOLA1	-	X	
cg09725915	2	70369583	Island		-	X	
cg09971754	16	89557657	Island	ANKRD11	+	X	
cg10546176	5	34929404	Island	DNAJC21	-	X	
cg10749792	7	56119218	Island	PSPH, CCT6A	-	X	
cg10776186	13	25875020	Island	NUPL1	-	X	Sex-dependent differentially methylated gene (McCarthy et al., 2014)
cg11065518	2	20763	S_Shore	MDH1B, FASTKD2	-	X	
cg11174255	4	1513259	N_Shore		+	X	
cg11240062	8	14436	Island		+	X	
cg11565911	12	72233249	N_Shore	TBC1D15	-	X	
cg11841231	2	20554		PARD3B	+	X	
cg12356266	8	99984350	N_Shore		-	X	
cg12611527	2	15725	Island		-	X	
cg12611723	9	139940	Island	NPDC1	-	X	
cg13230424	17	45930033	S_Shore	SP6	-	X	
cg13346869	8	37605517		LOC728024, ERLIN2	-	X	
cg14030268	10	11913	Island	PDZD8	-	X	
cg14373579	9	13345	Island	LOC100272217, FUBP3	-	X	
cg15148078	19	3480561	N_Shore	C19orf77	+	X	

(Continued)

TABLE 3 | Continued

Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Yusipov et al.	Cited in previous studies in relation to sex
cg15817705	1	20940	S_Shore		+	X	
cg16021159	1	57142074		PRKAA2	+	X	
cg16374663	15	41805031	Island	LTK	-	X	
cg17561891	7	86849173	Island	C7orf23	-	X	
cg17743279	7	92463268	Island	CDK6	-	X	
cg17887478	17	7486551	Island	MPDU1	-	X	
cg18001427	21	30391784	S_Shore	RWDD2B	-	X	
cg18721420	19	15121913	Island	CCDC105	-	X	
cg19292062	20	524344	Island	CSNK2A1	-	X	
cg19311244	4	77341912	N_Shore		-	X	
cg19864758	20	17206720	Island	PCSK2	-	X	
cg20050113	2	103236861	S_Shore	SLC9A2	-	X	Sex-dependent differentially methylated gene (McCarthy et al., 2014)
cg20432211	4	77342104	Island		-	X	
cg22105158	19	3480672	N_Shore	C19orf77	+	X	
cg22266749	4	110223	Island	COL25A1	+	X	
cg22345911	17	80231263	Island	CSNK1D	-	X	
cg22794378	14	89029563	Island	ZC3H14	-		
cg22799420	14	102028994	Island	DIO3	-	X	Sex-dependent regulation and functions (Sittig et al., 2011; Kim et al., 2019; Stohn et al., 2019; Stone et al., 2019)
cg22889142	19	58862398	Island	NCRNA00181, A1BG	-	X	Female-specific gene expression in liver (Gardmo and Mode, 2006; Conforto et al., 2012)
cg23001456	17	2615074	Island	KIAA0664	-	X	
cg23719534	15	10109	Island		-		
cg23880736	4	582172	Island		+	X	
cg24016844	1	11150	Island	C1orf103	+	X	
cg24126849	4	581937	N_Shore		+	X	
cg24158363	17	73401717	Island	GRB2	-	X	
cg24717799	15	83680832	S_Shore	C15orf40	-	X	
cg24990494	13	32520050		EEF1DP3	+		
cg25584814	19	345306	Island	MIER2	-	X	
cg25726513	4	1340596	Island	KIAA1530	-	X	
cg26172013	20	32031452	Island	SNTA1	-	X	
cg26516287	7	12629275		SCIN	-	X	
cg26612727	17	38024636	Island	ZPBP2	-	X	Sex-dependent DNA methylation (Ho et al., 2018)
cg27645294	17	21795257			-	X	

in CpG islands and enriched in the other genomic contexts, a significant enrichment in CpG islands was found for AD-DMPs identified in FC (**Supplementary File 7**). GO analysis returned significant results only in TC, where pathways related to synapse organization and function were found (**Supplementary File 7**).

Correlation analysis of effect sizes between the four brain regions highlighted a distinctive pattern in CRB respect to FC, TC, and ERC, while the correlation was higher between TC and ERC (**Figure 2C**). Accordingly the intersection between AD-DMPs in the 4 brain regions did not return common probes, while 29 probes (mapping in 23 genes) and 8 probes (mapping in 6 genes) were identified by intersecting TC and ERC or FC and TC, respectively (**Figure 2F** and **Table 5**). The probe cg12163800, mapping in Rhomboid 5 Homolog 2 (RHBDF2) gene, was significantly hypermethylated in FC, TC, and ERC from AD patients. A comparison with AD-associated probes retrieved

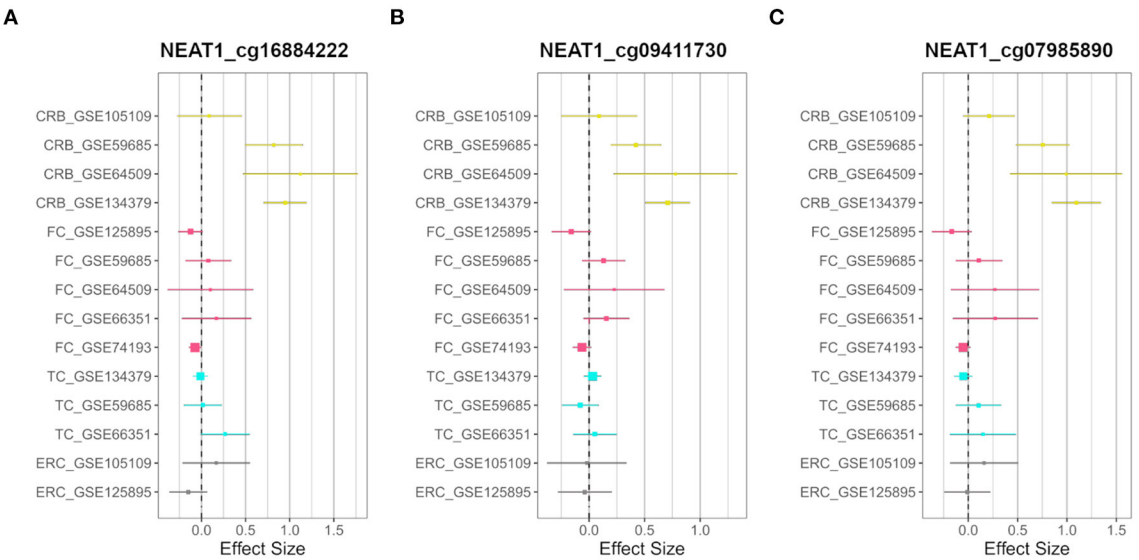

FIGURE 3 | CRB-specific sex-associated DNAm of *NEAT1* gene. Forest plots of three CRB-specific sDMPs mapping in *NEAT1*gene: **(A)** cg16884222, **(B)** cg09411730, **(C)** cg07985890. For each probe, effect sizes from the datasets used for our meta-analysis are reported, dividing them according to the four brain regions (CRB, yellow; FC, magenta; TC, cyan; ERC, gray).

in a recent meta-analysis (Smith R. G. et al., 2020) is also reported in **Table 5**.

Confirmation of Sex- and Age-Associated DNAm Changes in AD Subjects

We investigated whether the sDMPs and aDMPs identified above in the different brain regions from healthy controls were confirmed also in AD patients. To this aim, we evaluated their association with sex (correcting for age and estimated neuron/glia proportion) or with age (correcting for sex and estimated neuron/glia proportion) considering DNAm data from AD samples, and performed a meta-analysis in each brain region. The effect sizes obtained in AD were highly correlated with those previously obtained in healthy controls (**Supplementary Figure 5**). This correlation was slightly lower for aDMPs, which is expected considering that in most datasets the age range tends to be narrower for AD samples compared to healthy controls. Interestingly, also in AD samples we found an enrichment of sDMPs on chromosome 19 (data not shown). Collectively, these results indicate that sex- and age-dependent DNAm patterns are largely reproduced in AD samples.

The Relationship Between Sex- and Age-Associated DNAm Changes and AD Epigenetic Remodeling

We explored whether AD-associated DNAm changes were related to sex- and age-specific brain DNAm patterns occurring in physiological conditions, identified in the analyses described above.

In each brain region, we intersected the AD-DMPs and sDMPs in order to identify AD&sDMPs, i.e., probes that have basal differential DNAm between the two sexes and are also affected by AD. The intersection did not result in any probe for all the regions except that for TC, where we found 23 AD&sDMPs, mapping in 16 genes and corresponding to only 0.4% of AD-DMPs in TC (Fisher's Exact Test p-value >0.05; **Figure 4** and **Supplementary Files 1**, **6**). Moreover, AD-by-sex interaction analysis yielded no significant probes in any region.

To further explore the epigenetic relationship between sex and AD, we extended our analysis to probes located on sex chromosomes and focused on AD datasets in which their DNAm values were available (Section Materials and Methods). For each dataset, we considered males and females separately, we repeated the EWAS for AD-associated DNAm and we performed the meta-analysis within each brain region. We then searched for significant AD-DMPs located on the X or Y chromosomes. This analysis returned only few probes: four X-linked DMPs were found in TC when males with and without AD were compared, while one X-linked probe was found in male ERC (**Supplementary File 6**).

Similarly, we explored whether AD-DMPs occur in probes whose DNAm varies during physiological aging (AD&aDMPs). The intersection between AD-DMPs and aDMPs highlighted 7, 456, 4, and 0 probes in FC, TC, ERC, and CRB, respectively (**Figure 4**). The proportion of AD&aDMPs was higher than expected by chance in FC, TC, and ERC (Fisher's Exact Test p-value <0.05; odds ratio of 15.9, 3.8, and 95 in FC, TC, and ERC, respectively). We found that 87% of AD&aDMPs in TC are concordant for the effect size sign between aDMPs and AD-DMPs, while this percentage reached 100% in FC and ERC. Notably, the four AD&aDMPs found in ERC (cg11823178, cg03169557, cg25018458, and cg22090150) were also found in TC (**Table 6**). Also the intersection between AD&aDMPs in TC

TABLE 4 | aDMPs resulting from cross-region analysis.

Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Yusipov et al.	Cited in previous studies in relation to age
cg00292135	7	156433068	Island	C7orf13, RNF32	+	X	
cg04090392	15	83952774	Island	BNC1	+	X	Testicular premature aging (Li J. Y. et al., 2020)
cg06639320	2	106015739	Island	FHL2	+	X	Epigenetic changes in aging (Garagnani et al., 2012; Steegenga et al., 2014; Bacos et al., 2016; Kananen et al., 2016; Bacalini et al., 2017; Spólnicka et al., 2018b)
cg06942814	7	27170819	S_Shore	HOXA4	+	X	Epigenetic dysregulation in progeroid syndrome (Maierhofer et al., 2019)
cg07303143	3	44803452	Island	KIAA1143, KIF15	+	X	
cg07525420	10	131761181	Island	EBF3	+	X	
cg07922606	6	26225389	Island	HIST1H3E	+		Regulation of age-dependent gene expression (Crossland et al., 2017)
cg11614451	3	160167729	Island	TRIM59	+		Epigenetic changes in aging (Spólnicka et al., 2018a,b; Wezyk et al., 2018)
cg12373771	22	17601381	Island	CECR6	+	X	
cg13327545	10	22623548	Island		+	X	
cg14020846	14	103674272	Island		+	X	
cg14556683	19	15342982	Island	EPHX3	+	X	
cg15243034	11	77907656	Island	USP35	+	X	
cg15341124	14	102027734	Island	DIO3, MIR1247	+	X	Age-dependent expression (McCann and Ames, 2011; Kim et al., 2014; White et al., 2015; Mikovic et al., 2018; Wang et al., 2020)
cg15611336	15	75248496	Island	RPP25	+	X	
cg16295725,	4	10459219	Island	ZNF518B	+	X	Pancreatic aging (Bacos et al., 2016; Bou Sleiman et al., 2020)
cg23995914		10459228					
cg16867657	6	11044877	Island	ELOVL2	+	X	Epigenetic changes in aging (Garagnani et al., 2012; Steegenga et al., 2014; Rönn et al., 2015; Bacalini et al., 2017; Slieker et al., 2018; Spólnicka et al., 2018b; Sturm et al., 2019; Chao and Skowronska-Krawczyk, 2020; Chen et al., 2020; Li X. et al., 2020)
cg16969368	17	57642752	Island	DHX40	+	X	
cg18008766	2	38978896	S_Shore	SFRS7	+	X	
cg18240400	10	46168597	Island	ANUBL1	+	X	
cg18473521	12	54448265	S_Shore	HOXC4	+	X	
cg19399220	19	10527588	Island		+	X	
cg20591472	1	110008990	Island	SYPL2	+	X	
cg24079702	2	106015771	Island	FHL2	+	X	Epigenetic changes in aging (Garagnani et al., 2012; Steegenga et al., 2014; Bacos et al., 2016; Kananen et al., 2016; Bacalini et al., 2017; Spólnicka et al., 2018b)
cg24567591	16	3931229	Island	CREBBP	+	X	Memory performance in elderly (Barral et al., 2014)
cg24903144	10	102509268	Island	PAX2	+	X	Retina aging (Mansour et al., 2008)
cg26092675	6	26225258	N_Shore	HIST1H3E	+	X	Regulation of age-dependent gene expression (Crossland et al., 2017)

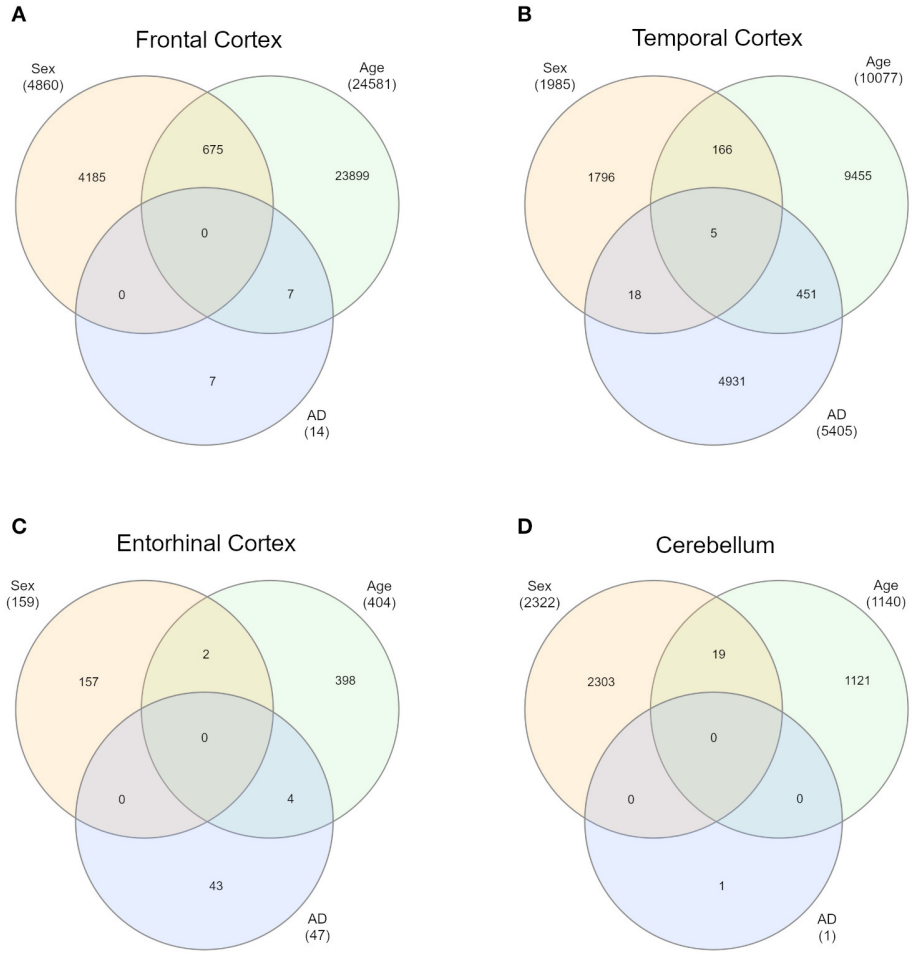

FIGURE 4 | Intersections of sex-, age-, and AD-associated probes in each of the four brain regions. Venn diagrams depict the intersection between sDMPs, aDMPs, and AD-DMPs in FC **(A)**, TC **(B)**, ERC **(C)**, and CRB **(D)**.

and FC returned four common probes (cg01463828, cg04874795, cg22962123, and cg07061298; **Table 6**). **Figure 5** reports DNAm values of cg11823178 (ANK1) and cg22962123 (PDLIM2) in TC from GSE134379 dataset as an example of CpG sites displaying a positive association of DNAm with age and hypermethylated in AD.

Finally, it is worth to note that TC is the only brain region in which we found probes at the intersection between aDMPs, sDMPs, and AD-DMPs (AD&a&sDMPs; **Figure 4B**). The five probes (cg20225999, cg03951603, cg08820801, cg22263793, cg10828284; **Table 7**) were all hypermethylated in males and with aging; three of them (cg20225999, cg08820801, cg10828284) were further hypermethylated in AD.

Contribution of 5-hydroxymethylcytosine to the Epigenetic Changes Across Sex, Age, and AD

All the analyses reported above are based on microarray data from BS converted DNA. BS treatment does not allow to distinguish between 5mC and 5hmC, another epigenetic mark which plays an important role especially in the brain (Kriaucionis and Heintz, 2009; Lunnon et al., 2016). On the contrary, the combination of BS with oxBS treatment allows discriminating the levels of 5mC, 5hmC, and 5uC in DNA (Booth et al., 2012). One of the datasets that we used in our meta-analysis for sex-, age-, and AD-associated epigenetic changes (GSE105109) includes microarray results from matched BS- and OxBS-treated ERC and CRB samples. We calculated the levels of 5mC, 5hmC, and 5uC (Section Materials and Methods) in this dataset and we analyzed them for the association with sex (in healthy subjects), with age (in healthy subjects), and with AD (comparing AD and healthy subjects). This analysis did not return any significant probe, neither in ERC nor in CRB. We then considered the lists of sDMPs, aDMPs, and AD-DMPs identified in the meta-analysis of ERC and CRB datasets, and used GSE105109 data to investigate the contribution of 5mC, 5hmC, and 5uC to the observed epigenetic changes. **Supplementary Figure 6** reports the correlation between the effect size values resulting from the meta-analysis of sex, age, and AD, and the effect size values

TABLE 5 | AD-DMPs resulting from cross-region analysis.

Intersection	Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Smith et al.	Cited in previous studies in relation to AD
TC ∩ ERC	cg00851830	14	100201016	N_Shelf		+		
	cg03169557	16	89598950		SPG7	+	X	Retinal nerve fiber layer loss; AD DMP (Wiethoff et al., 2012; Li Q. S. et al., 2020)
	cg03183618	2	134964228			+		
	cg04658038	17	64800166		PRKCA	+		Synaptic degeneration (Wang et al., 2010; Alfonso et al., 2016; Maphis et al., 2017)
	cg05066959 cg11823178	8	41519308 41519399		ANK1, MIR486	+	X	Epigenetic changes in AD; involved in memory loss (De Jager et al., 2014; Lord and Cruchaga, 2014; Lunnon et al., 2014; Chi et al., 2016; Mastroeni et al., 2017; Gasparoni et al., 2018; Higham et al., 2019; Blanco-Luquin et al., 2020; Li Q. S. et al., 2020)
	cg05397697	14	90042217		PRO1768, FOXN3	+		
	cg05417607	17	1373605	N_Shore	MYO1C	+	X	
	cg05810363,	17	74475270	Island	RHBDF2	+	X	Epigenetic changes in AD (De Jager et al., 2014; Lord and Cruchaga, 2014; Zou et al., 2019; Li Q. S. et al., 2020)
	cg12163800,		74475355					
	cg12309456		74475402					
	cg06653632	12	129281444	S_Shore	SLC15A4	+		
	cg06753513	17	3977385		ZZEF1	+		
	cg07012687	17	80195180	Island	SLC16A3	+		
	cg07571519	10	73472315		C10orf105, CDH23	+		Expression and epigenetic changes in AD (De Jager et al., 2014; Lord and Cruchaga, 2014; Humphries et al., 2015; Hu et al., 2018)
	cg09123026	17	74480528		RHBDF2	+		Epigenetic changes in AD (De Jager et al., 2014; Lord and Cruchaga, 2014; Zou et al., 2019; Li Q. S. et al., 2020)
	cg13851211	16	50321678		ADCY7	+		
	cg14025831	20	3873404	S_Shelf	PANK2	+		
	cg14761246	3	182968758	N_Shelf	MCF2L2	+		
	cg14798745	4	184315677	N_Shelf		+		
	cg18102633	19	17487776	N_Shore	PLVAP	+		
	cg18456331	10	77188318	N_Shelf		+		
	cg18923906	10	82225771		TSPAN14	+		
	cg20148994	7	130125585	N_Shore	MEST	+		
	cg21221455	15	63342288	S_Shore	TPM1	+		
	cg22090150	17	4098227		ANKFY1	+	X	
	cg22656126	17	1637206	Island	WDR81	+		
	cg25018458	17	980014	N_Shore	ABR	+	X	Hearing loss (Irimajiri et al., 2005; Oh et al., 2010; O'Leary et al., 2017; Hacohen-Kleiman et al., 2019; Liu et al., 2020)
	cg27630153	16	88845038	Island	FAM38A	+		
FC ∩ TC ∩ ERC	cg12163800	17	74475355	Island	RHBDF2	+	X	Epigenetic changes in AD (De Jager et al., 2014; Lord and Cruchaga, 2014; Zou et al., 2019; Li Q. S. et al., 2020)

(Continued)

TABLE 5 | Continued

Intersection	Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Smith et al.	Cited in previous studies in relation to AD
FC ∩ TC	cg01463828	8	22446721		PDLIM2	+	X	
	cg02317313	12	122	Island	LOC338799	+	X	
	cg04874795	16	86477638			-	X	
	cg07061298	7	27153847	N_Shore	HOXA3	+	X	Epigenetic changes in AD (Gasparoni et al., 2018; Hernández et al., 2018; Smith et al., 2018; Li Q. S. et al., 2020)
	cg12163800	17	74475355	Island	RHBDF2	+	X	Epigenetic changes in AD (De Jager et al., 2014; Lord and Cruchaga, 2014; Zou et al., 2019; Li Q. S. et al., 2020)
	cg22962123	7	27153605	Island	HOXA3	+	X	
	cg26022064	7	98739782	N_Shore	SMURF1	+	X	Neural necroptosis and Hirano bodies (Makioka et al., 2014; Shao et al., 2018)
	cg26199857	12	54764265	Island	ZNF385A	+		
FC ∩ ERC	cg12163800	17	74475355	Island	RHBDF2	+	X	
	cg13076843		74475294					

TABLE 6 | AD&aDMPs resulting from cross-region intersections.

Intersection	Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Smith et al.	Yusipov et al. (Bonf. Corrected aDMPs)
ERC ∩ TC	cg11823178	8	41519399		ANK1	+	X	X
	cg03169557	16	89598950		SPG7	+	X	
	cg25018458	17	980014	N_Shore	ABR	+	X	
	cg22090150	17	4098227		ANKFY1	+	X	
FC ∩ TC	cg22962123	7	27153605	Island	HOXA3	+	X	X
	cg07061298	7	27153847	N_Shore	HOXA3	+	X	
	cg04874795	16	86477638			-	X	X
	cg01463828	8	22446721		PDLIM2	+	X	

obtained in GSE105109 dataset using 5mC, 5hmC, and uC values in the association analysis. In both ERC and CRB, sDMPs and AD-DMPs showed high correlation between BS (5mC+5hmC) results and oxBS (5mC) results, while the correlation with 5hmC results was low. A similar trend was observed for aDMPs in CRB. This indicates that 5mC is the main contributor to the epigenetic changes observed for the sDMPs and the AD-DMPs in ERC and CRB, and for the aDMPs in CRB. On the contrary, for ERC aDMPs, BS-effect sizes were similarly correlated with 5mC- and 5hmC-effect sizes, indicating that both the epigenetic marks are remodeled during aging in this brain region. Furthermore, age-associated changes in 5mC and 5hmC were likely to involve different probes, as 5mC and 5hmC effect sizes were not clearly correlated.

DISCUSSION

Sex and age are among the major risk factors for AD. In this paper, we performed a meta-analysis of DNAm changes that are associated to sex and aging in four brain regions (FC, TC, ERC, CRB) and we evaluated whether they contribute to the epigenetic alterations that have been widely described in AD. Our main findings are discussed in the following paragraphs.

Sex-Dependent DNAm Differences Tend to Be Shared Between Brain Regions, With Few Exceptions

To date some studies have reported DNAm sex differences in human brain, mainly focusing on frontal cortex (Xu et al., 2014; Spiers et al., 2015; Masser et al., 2017; Perzel Mandell et al., 2020) with few exceptions (Xia et al., 2019). Our meta-analysis confirms the presence of autosomic probes with differential methylation between males and females in all the brain regions. These probes preferentially map in CpG islands and shores suggesting their involvement in the regulation of sex-specific gene expression in brain (Xu et al., 2014). Surprisingly, in all the brain regions we found an enrichment of sDMPs in chromosome 19. This observation is difficult to be explained but a similar result

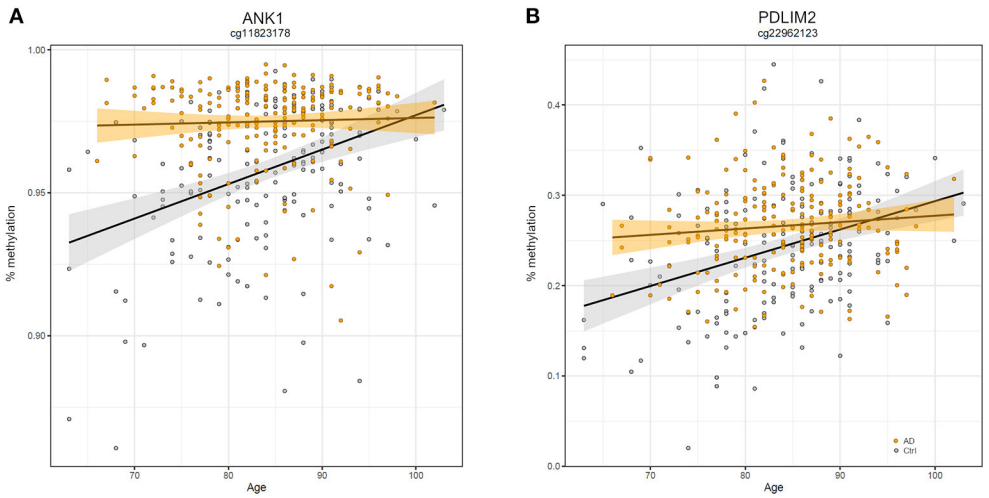

FIGURE 5 | Scatter plots of *ANK1* and *PDLIM2* DNAm according to age and disease. Scatter plots of methylation values of cg11823178 within *ANK1* **(A)** and of cg22962123 within *PDLIM2* **(B)** in TC from GSE134379 dataset. Healthy subjects are colored in gray while AD patients are in orange. Regression lines and confidence intervals within each group are reported.

TABLE 7 | Probes resulting from the intersection between aDMPs, sDMPs, and AD-DMPs in TC.

Probe	Chr	MAPINFO	Relation	Gene	Effect size direction	Smith et al.	Yusipov et al.	Yusipov et al.
cg20225999	2	218843435	N_Shore		+	X		X
cg03951603	15	89903565	Island		-		X	
cg08820801	19	39465821	N_Shore	FBXO17	+		X	
cg22263793	19	42501398	Island		-		X	
cg10828284	22	50528333	Island	MOV10L1	+		X	

was observed in a precedent study on sex-associated DNAm differences across childhood in whole blood (Suderman et al., 2017). Chromosome 19 has the highest content of CpG sites and genes in the genome (Grimwood et al., 2004), and seems to be involved in the process of X chromosome inactivation (Migeon et al., 2017).

Sex specific DNAm tended to be reproducible across the brain regions and 77 CpGs resulted from the cross-region intersection. Among them there are sDMPs mapping in genes that have been already associated to sex differences in brain physiology and pathology, like Par-3 Family Cell Polarity Regulator Beta (*PARD3B*) (Phillips et al., 2019), DEAF1 Transcription Factor (*DEAF1*) (Luckhart et al., 2016), and Iodothyronine Deiodinase 3 (*DIO3*) (Stohn et al., 2019) genes. Most of these 77 probes were previously reported as differentially methylated between males and females also in previous meta-analysis on blood (McCarthy et al., 2014; Yusipov et al., 2020).

In addition, we found few examples of sDMPs specific for a brain region. The most notable example is in cerebellum and maps in *NEAT1*. *NEAT1* is a ubiquitously expressed long non-coding RNA (lncRNA) involved in a plethora of neurospecific processes such as brain development and aging (An et al., 2018; Pereira Fernandes et al., 2018; Salvatori et al., 2020). Recent transcriptomic studies on human central nervous system revealed altered *NEAT1* levels in AD (Spreafico et al., 2018), PD (Simchovitz et al., 2019), and in schizophrenia (Katsel et al., 2019).

DNAm Tends to Be Differently Remodeled During Aging According to the Brain Region

Several studies have analyzed age-associated changes in DNAm in brain, both comparing fetal vs. adult brains and analyzing methylation profiles across adulthood (Hernandez et al., 2011; Horvath et al., 2012; Numata et al., 2012; Day et al., 2013; Jaffe et al., 2016; Gasparoni et al., 2018; Price et al., 2019). Our meta-analysis shows that during aging there is an increase in methylation at specific loci, accordingly to previously published data on blood (Xiao et al., 2016; Yusipov et al., 2020) and brain (Hernandez et al., 2011). As previously reported by Hernandez et al. (2011), also our results support the involvement of brain aDMPs in GO related to developmental processes and morphogenesis. Furthermore, our meta-analysis confirms and extends the observation that the epigenome is differently remodeled during aging across brain regions (Hernandez et al.,

2011). In particular we observed that age-associated DNAm patterns are similar in TC and FC, while they are distinct in ERC and CRB. CRB was previously described to undergo a peculiar epigenetic aging, which was decelerated according to Horvath's epigenetic clock (Horvath et al., 2015).

The large fraction (93%) of the 28 aDMPs emerged from our cross region analysis was found also in aging studies on blood (Yusipov et al., 2020). Among them there are probes mapping in Four And A Half LIM Domains 2 (*FHL2*) and ELOVL Fatty Acid Elongase 2 (*ELOVL2*) genes, previously reported as age-associated in a large number of studies on several tissues (Garagnani et al., 2012; Slieker et al., 2018) including sorted neuron and glia cells (Gasparoni et al., 2018). According to what discussed above and to previous results (Bacalini et al., 2017; Slieker et al., 2018) the effect size of *ELOVL2* probe cg16867657 was lower in CRB respect the other regions, but still significant in our meta-analysis. Elovl2 is an enzyme involved in the elongation of fatty acids and its functional role in aging has been recently suggested (Chao and Skowronska-Krawczyk, 2020).

Sites With Sex-Dependent DNAm Are Similarly Modulated During Aging in Males and Females

Previous studies in mice and humans suggested that, while sex-differences in DNAm at certain CpG sites are maintained during life, other CpG sites show sexually divergent aging patterns, i.e., they have a different response to aging in males and females (Masser et al., 2017). Our meta-analysis supports the fact that sDMPs have a high propensity to be modulated during aging, as the number of probes resulting from the intersection of sDMPs and aDMPs is higher than expected in all the four brain regions. However, we found only few probes with significant age-by-sex interaction, indicating similar rather than diverging changes in DNAm in males and females aging. The discrepancy between our results and previous findings can be due to different reasons: for example, while Masser et al. considered only one dataset including frontal cortex data, here we meta-analyzed several datasets using selective criteria of concordance between all datasets from the same brain region; furthermore, we applied a filtering step that removed potentially ambiguous probes, thus reducing the potential overlap with Masser's results. Our results are more similar to what reported by two independent studies in blood (McCartney et al., 2019; Yusipov et al., 2020) that showed that only a small fraction of CpGs have significant age-by-sex interaction. Further studies on larger cohorts are needed to better describe sex-dependent DNAm patterns during brain aging.

Epigenetic Changes in AD Are Enriched in Sites That Show Age-Dependent DNAm

A recent meta-analysis on EWAS studies identified 220 CpGs associated with AD neuropathology, shared by brain cortical cortex regions but not by CRB (Smith et al., 2019). The paper by Smith et al. included several datasets that we used also in our meta-analysis, with the exception of GSE125895 and GSE109627, while we did not have access to the ROS/MAP and RBD DNAm data. Furthermore, while Smith et al. considered the association with Braak stage, here we used the disease as a binary trait (affected/unaffected). Despite these differences, our results largely overlap with those previously reported. In particular, we did not find AD-related probes common to all the four brain regions that we investigated, with CRB DNAm less affected by the pathology. On the contrary, a subset of sites was shared between FC, TC, and ERC, and about 50% of these probes overlap with published data. These probes map within genes whose epigenetic deregulation has been largely documented in AD, including *ANK1*, *RHBDF2,* and *HOXA3*. On the contrary, we did not find any overlap when comparing our results on AD brain with CpG sites identified in AD patients' blood (Roubroeks et al., 2020), confirming that the pathology differently affects the two tissues as recently reported (Wei et al., 2020).

We did not find a significant overlap between AD-DMPs and sDMPs, nor significant interaction effects between sex and AD. Overall these results suggest that AD does not predominantly insist on autosomic sites with sex-specific DNAm. Similarly, when we repeated our analysis including probes on X and Y chromosomes (analyzing males and females separately) we found limited evidence of differential DNAm between AD and controls in sex chromosomes. Collectively these results suggest that no profound sex-associated DNAm remodeling occurs in AD. However, we cannot rule out that more subtle epigenetic differences exist, both on autosomes and sex chromosomes. It is possible that these differences did not emerge from our meta-analysis, due to the stringent selection criteria that we applied or to the small sample sizes when males and females were considered separately. Further studies should investigate possible epigenetic contributions to the different AD risk between the two sexes.

Conversely, our data show that in FC, TC and ERC, AD-related epigenetic modifications are significantly enriched in probes whose DNAm varies with age. Strikingly, we found a high concordance between the direction of DNAm changes (hyper or hypo-methylation) in AD&aDMPs, indicating that a subset of age-associated DNAm changes is exaggerated in AD. In TC, AD&aDMPs included probes mapping in *ANK1*, and it is worth to note that the down-regulation of Ank2 (ANK1 human ortholog gene) in *Drosophila* has been associated to memory loss, neuronal dysfunction and shortened lifespan in a recent report (Higham et al., 2019). Other interesting genes emerged from our analysis. Paraplegin (SPG7) mutation leads to shortened lifespan, environmental stress. and muscular and neuronal degeneration in *Drosophila* (Pareek et al., 2018). Mov10 Like RISC Complex RNA Helicase 1 (MOV10L1) is a putative germline-specific RNA helicase whose expression has been recently reported to be tightly correlated with brain development, aging and AD neurodegeneration (Skariah et al., 2017; Srinivasan et al., 2020).

Overall, these results support a geroscience view (Kennedy et al., 2014; Sierra, 2020) according to which AD can be considered a deviation of the physiological aging trajectories toward accelerated aging. Epigenetic age acceleration was previously reported in AD neurons, where a pronounced loss of CpH methylation was found at enhancers, similar to what observed in aging, and in bulk prefrontal cortex, where epigenetic age calculated by Horvath's clock was positively associated with

neuritic plaques and amyloid load (Levine et al., 2015). It will be interesting to know whether similar results will be obtained using the recently published epigenetic clock optimized for brain tissues (Shireby et al., 2020).

Strengths, Limitations, and Conclusions

To the best of our knowledge, this is the first report in which sex-, age-, and AD-related DNAm changes are systematically assessed using the same analytical approach. We used stringent selection criteria that enabled to select only probes with concordant DNAm changes in the different datasets. Furthermore, we considered multiple brain regions and reported similarities and differences in their epigenetic remodeling. Previous studies showed that DNAm patterns differ between brain regions and that they may play a role in brain development and functional specialization (Ladd-Acosta et al., 2007; Rizzardi et al., 2019). These "baseline" DNAm differences can mediate disease mechanisms that are specific for certain brain regions (Rizzardi et al., 2019), and can be further modified across lifespan and in response to pathological conditions. Accordingly, brain areas are differently affected during aging and/or in AD onset and progression (Peters, 2006; Coupé et al., 2019), and also other molecular layers like transcriptomics and proteomics show region-specific changes (Patel et al., 2019; Xu et al., 2019).

On the other side, our study has some limitations. The datasets that we meta-analyzed largely vary in size and age range of the assessed subjects, an important aspect for the identification of aDMPs. Moreover, our meta-analysis included data on BS-treated DNA and it was therefore not possible to distinguish 5mC from 5hmC, an epigenetic modification that contributes to both brain function and neurodegeneration (Coppieters et al., 2014; Ellison et al., 2017; Lardenoije et al., 2019; Smith et al., 2019). The analysis of the dataset in which 5mC and 5hmC were distinguishable (thanks to the simultaneous analysis of BS- and oxBS-treated DNA) suggested that the contribution of 5hmC to sDMPs and AD-DMPs tended to be small. A recent study showed that in fetal brain autosomal 5hmC levels did not differ between males and females (Spiers et al., 2017), in accordance to our results. Global changes in 5hmC have been reported to occur in AD (Chouliaras et al., 2013; Condliffe et al., 2014; Coppieters et al., 2014), while microarray-based genome wide studies identified a limited set of CpG sites whose hmC levels are associated to the disease (Lardenoije et al., 2019; Smith et al., 2019). For example, Smith et al. reported that AD-associated hypermethylation of *ANK1* detected on BS-treated DNA is not due to an increase in 5hmC levels, which on the contrary decreased in the disease (Smith et al., 2019). Similarly, in our analysis of AD in ERC most of the significant associations were due to changes in 5mC and we did not observe an evident co-variation of 5mC and 5hmC. On the contrary, when considering aDMPs in ERC we found a contribution of both 5mC and 5hmC, and the two epigenetic marks tended to involve different CpG sites. Age-associated increase in 5hmC levels has been previously reported (Chouliaras et al., 2012). Although potentially interesting, these results are based on only one dataset, and the analysis of the coordinated regulation of brain 5mC and 5hmC across sex, age, and AD deserves further studies. Another limitation of our study is that the datasets that we meta-analyzed were based on bulk brain tissues. Although all the analyses were corrected for neuron/glia proportions predicted from DNAm data, we cannot exclude that the observed sex-, age-, and AD-associated DNAm changes are at least in part driven by changes in brain cells composition that occur in physiological and pathological conditions. For example, Gasparoni et al. reported that *ANK1* deregulation in AD is specific for glial cells (Gasparoni et al., 2018), a finding further supported by gene expression studies (Mastroeni et al., 2017), and that the epigenetic profiles of neurons and glia are differently modulated during aging. Notwithstanding, our results suggest that the (cell-specific) age-associated remodeling of DNAm is not just a confounding factor for the epigenetic deregulation observed in AD, but on the contrary, it is the predisposing *milieu* in which AD pathogenetic mechanisms are established.

In conclusion, we suggest that age-associated DNAm patterns concur to the epigenetic deregulation observed in AD, providing new insights on how advanced age enables neurodegeneration.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CPe, CPi, CS, MI, DM, RL, CF, PC, PG, and MGB contributed to the conception and design of the study. CPe, CS, IY, KK, DD, and MGB organized the datasets. CPe, CPi, CS, FR, IY, KK, AK, and MGB performed the statistical analysis. MGB, CPe, and CPi wrote the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.639428/full#supplementary-material

Supplementary Figure 1 | Overview of the meta-analysis. For simplicity, the age-by-sex and AD-by-sex interaction analyses are not reported, but they were performed using the same pipeline illustrated in this scheme.

Supplementary Figure 2 | Manhattan plots of sDMPs in the four brain regions. The figure displays the Manhattan plots resulting from the meta-analysis of sex-associated probes in FC **(A)**, TC **(B)**, ERC **(C)**, and CRB **(D)**. Significant sDMPs are marked with dark color. Scale change across 50 is indicated by an axis break.

Supplementary Figure 3 | Manhattan plots of aDMPs in the four brain regions. The figure displays the Manhattan plots resulting from the meta-analysis of age-associated probes in FC **(A)**, TC **(B)**, ERC **(C)**, and CRB **(D)**. Significant aDMPs are marked with dark color. Scale change across 50 is indicated by an axis break.

Supplementary Figure 4 | Manhattan plots of AD-DMPs in the four brain regions. The figure displays the Manhattan plots resulting from the meta-analysis of AD-associated probes in FC **(A)**, TC **(B)**, ERC **(C)**, and CRB **(D)**. Significant AD-DMPs are marked with dark color.

Supplementary Figure 5 | Confirmation of sDMPs and aDMPs in AD patients. The scatter plots report, for the sDMPs and aDMPs identified in each tissue in healthy subjects, the effect sizes obtained in healthy subjects against the effect sizes resulting from the meta-analysis in AD patients. Pearson Correlation coefficient is reported in each plot.

Supplementary Figure 6 | Contribution of 5hmC to the epigenetic changes across sex, age, and AD. Correlation plots of the effect sizes of sDMPs, aDMPs, and AD-DMPs identified in ERC and CRB, calculated using BS values (5mC+5hmC), oxBS values (5mC), BS-oxBS values (5hmC), and 1-BS values (5uC). Absolute correlation values are reported.

Supplementary File 1 | Significant sDMPs in the four brain regions. The tables report the lists of sDMPs for each brain region (FC, TC, ERC, and CRB). Probes resulting from the analysis of cross-region and region-specific sDMPs are indicated by a cross, together with the probes that are in common with aDMPs or AD-DMPs found in the same region.

Supplementary File 2 | Enrichment analysis of sDMPs. The tables report: (1) the results of Fisher's test on genomic distribution of sDMPs for each brain region, considering genomic context and chromosomal location. Significant results (p-value <0.05) are colored in green or red if depleted or enriched, respectively. (2) the results of GO pathway enrichment analysis, after REViGO filtering. Only the significant results (adjusted p-value <0.01) for each brain region are reported.

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Supplementary File 5 | Probes with significant age-by-sex interaction in the four brain regions. The tables report the lists of probes with significant age-by-sex interaction in each brain region (FC, TC, ERC, and CRB).

Supplementary File 6 | Significant AD-DMPs in the four brain regions. The tables report the lists of AD-DMPs for each brain region (FC, TC, ERC, and CRB). Probes resulting from the analysis of cross-region and region-specific AD-DMPs are indicated by a cross, together with the probes that are in common with sDMPs or aDMPs found in the same region. The sheet "Sex Chromosomes" reports the significant AD-DMPs identified on sex chromosomes when analyzing males and females separately.

Supplementary File 7 | Enrichment analysis of AD-DMPs. The tables report: (1) the results of Fisher's test on genomic distribution of AD-DMPs for each brain region, considering genomic context and chromosomal location. Significant results (p-value <0.05) are colored in green or red if depleted or enriched, respectively. (2) The results of GO pathway enrichment analysis, after REViGO filtering. Only the significant results (adjusted p-value <0.01) for each brain region are reported.

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Video Games in ADHD and Non-ADHD Children: Modalities of Use and Association With ADHD Symptoms

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Video game addiction in young children is relevant, but it is especially important for children with ADHD. In order to obtain more data about the use of video games by Canadian children, and in particular by ADHD children, we explored the modalities of use (playtime, addiction score and usage by age) and compared them between ADHD and non-ADHD children. We then examined associations between addiction and ADHD symptoms and explored innovative results about the gender impact. Our study was cross-sectional, multicenter in child psychiatrist departments, exploratory and descriptive. We recruited three groups of children aged 4–12 years: the ADHD Group, the Clinical-Control Group and the Community-Control Group. For each group, the material used consisted of questionnaires completed by one of the parents. Data collection took place from December 2016 to August 2018 in Montreal ($n = 280$). Our study highlighted a vulnerability in ADHD children: they would exhibit more addictive behaviors with respect to video games (Addiction score: 1.1025 in ADHD Group vs. 0.6802 in Community-Control Group) and prolonged periods of use. We also observed a correlation between the severity of ADHD symptoms and excessive use of video games ($p = 0.000$). Children with severe ADHD showed significantly higher addiction scores and, in a multiple regression analysis a combination of gender and ADHD explained the excessive use of video games.

Keywords: video game, ADHD, addiction, dependence, playtime, children

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INTRODUCTION

Children and adolescents report playing video games frequently, and we can see that this use is being reported at ever-younger ages (1). For example, 91% of children aged 2–17 are reported to play video games (2). New games on smartphones and tablets are rapidly being developed, many of which target young children including toddlers (3). The use of screens starts at an early age with more than 30% of children having used a tablet before the age of 2, and often for playing video

games. From about the age of 4, the computer has become an increasingly popular medium for children to play video games (4). When children get older, a vast array of devices are used such as consoles, computers, tablets and smartphones, both online and offline.

The period between 4 and 12 years is therefore an important stage where children are increasingly exposed to video games and as such represents a relevant developmental period to study factors linked to excessive or addictive use of video games. Studies tend to show that ∼2.0–5.5% of adolescents/young adults exhibit an addiction to video games (5). Multiple factors including the types of video games, personality characteristics and early exposure contribute to this addiction, but its origins are complex and gaps exist, particularly pertaining to such use by children (6).

In 2018, the World Health Organization (WHO) introduced Gaming disorder (GD) to the International Classification of Disease-11 (ICD-11) (7) and highlighted three symptoms: "impaired control over gaming, increasing priority given to gaming and continuation or escalation of gaming despite the occurrence of negative consequences" (8). Similarly, Internet gaming disorder (IGD) was included in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) in 2013, but it was categorized as requiring further study and not sufficiently well-established to be a part of the official classification of mental disorders for routine clinical use (9). IGD requires experiencing five or more of the following symptoms within a year: "preoccupation or obsession, withdrawal, tolerance, loss of control, loss of interest, continued overuse, deceiving, escape of negative feelings, functional impairment" (8). In this article, we will use the expression "video game addiction" which, in summary, shall be interpreted as a repeated use of video games that results in a significant impairment to an individual's social, family or professional life over a prolonged period of time.

The association between attention deficit hyperactivity disorder (ADHD) symptoms and video game addiction is observed among children and adolescents, but it is as yet not understood (10). The relationship between ADHD and the excessive use of video games may likely be bidirectional and needs to be clarified. In addition, most studies have been conducted among adolescents, and data for preschool children is almost non-existent apart from a 2018 study by Paulus et al. (11) ADHD is a risk factor for addiction in general (12), and it is the most frequent psychopathology in video game addiction (13). It is already listed in the DSM-5 as comorbid with Internet gaming disorder (9). However, the use of online games is mostly among adolescents and young adults and less in children who usually start with offline video games (4).

Hypotheses and Objectives

We've seen the major role that video games play in today's society, particularly among teenagers but also among increasingly younger children. Video gaming is typically a leisure activity, but its practice among some people can turn into an addiction and have negative consequences. Following an overview of the literature, we observed that the majority of studies on this topic are cross-sectional and prospective. These studies are conducted among teenagers and young adults without a control group and have seldom targeted children and only very rarely young (preschool or early primary school-aged) children. The scales used to measure video game addiction are highly variable and unvalidated. Furthermore, diagnosis of ADHD or other comorbidities is not generally confirmed through use of validated scales or by a specialized doctor [(child) psychologist, pediatrician].

In this context and with a view to adding to knowledge about video gaming and its potential risks in potentially vulnerable populations, we sought to compare, in an exploratory manner, children aged 4–12 from a clinical population (divided into two subgroups, ADHD and non-ADHD) with children aged 4–12 from the general population through use of standardized, validated scales. Based on our hypotheses, we expected to see an increase in addictive video gaming behaviors and in duration of video game use among children in the ADHD subgroup in comparison to the community population. Another of our hypotheses was that the intensity of ADHD symptomatology correlates positively with addictive behaviors.

In order to better understand the relationship between video game use/addiction and ADHD in preschool/school children, we conducted a descriptive and exploratory study with the following objectives:

(1) To determine the modalities of use of video games (playtime, addiction score and usage by age) in children with ADHD compared to children without ADHD.
(2) To examine the associations between video game addiction and ADHD symptoms.
(3) To explore the gender difference in video game use, the type of video games played by children with ADHD and the impact of parents on gaming.

METHODS

Participants

The participants were children aged 4–12 who comprised the three study groups. The first group was made up of children with ADHD (clinical group); the second was made up of children presenting one or more mental health diagnoses other than ADHD (clinical control group); and the third was made up of children from the general community (community control group). All participants' parents had to be able to speak and understand French. The only exclusion criteria were the inability to read, write and understand French, and the presentation of an intellectual handicap or active psychotic symptoms.

The age range of 4–12 was selected in order to more specifically target video gaming among school and preschool-aged children. It would be more difficult to direct our research questions at a broader age range in light of the inherent heterogeneous aspect already present in the children. With this in mind, the aspects evaluated as part of this study were not specific but rather related to individuals' general functioning.

Participants in the clinical and clinical control groups were recruited at the outpatient pediatric (ADHD and development) and child psychiatric (outpatient age 6–12, Tourette and age

0–5) clinics at the CHU Sainte-Justine, and the outpatient child psychiatric clinics at the Hôpital-Rivière-des-Prairies of the CIUSSS du Nord-de-l'Île-de-Montréal (CIUSSS NIM). These persons were recruited by a research assistant while waiting for an appointment in the waiting room at the outpatient pediatric or child psychiatric clinic. The assistant proposed participating in the study to all waiting patients and their parents and informed them about the project. The third group (community control group) was recruited at day camps in the Montreal, Laurentides and Lanaudière regions. Parents with their children were approached by a research assistant as they arrived at or left the day camp. The recruitment procedure followed was identical to that detailed above.

The exclusion criteria for parents and children were being unable to read, write and understand French. Participants ought not to have an intellectual disability or to be actively psychotic.

Study Design

The study was cross-sectional, multicentre (CHU Sainte-Justine, CIUSSS du Nord-de-l'Île-de-Montréal) in child psychiatric departments, exploratory and descriptive. The material used consists of questionnaires to be completed by one of the parents for each group. Data was collected between December 2016 and August 2018 in Montreal. This study is a first step toward evaluating this type of problem. The tools used consisted of validated questionnaires (three questionnaires described below in the Measures section) to be completed by one of the parents in one sitting (with a 30-min estimated completion time). After obtaining consent, a numbered envelope was given to each participant containing the questionnaires and a recruitment letter). This letter restated the main information concerning the research project and the project procedure and provided the telephone number of the research coordinator at the CHU Sainte-Justine in the event that participants needed additional information.

In the clinical group, in addition to the questionnaires, participants' medical records were consulted once by a research assistant to verify psychiatric diagnoses. All ADHD and/or psychiatric diagnoses were made by a child psychiatrist from the CHU Sainte-Justine or the CIUSSS NIM. Diagnoses were arrived at following a comprehensive, multidisciplinary assessment based on the validated diagnostic criteria from the reference diagnostic guide in psychiatry in North America, the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders).

Measures

The tools used consisted of three questionnaires completed by one of the parents in one sitting: the Sociodemographic Questionnaire with added questions on video game use among children and parents, the SDQ (Strengths and Difficulties Questionnaire) and the Questionnaire sur l'attention et l'ordinateur (questionnaire on attention and computers) (QUATTORD).

Sociodemographic Questionnaire With Added Questions on Video Game Use

General sociodemographic data were documented, including participant age, sex, current education level or last level achieved, family status, ethnic origin, etc., as well as the time of year of questionnaire completion. A standard sociodemographic questionnaire was used to collect this information supplemented by questions about screen time and, in particular, video game use. The added questions included questions about total screen time devoted to video gaming or to watching movies or television during the week and on weekends/holidays, frequency of use by game type and number of electronic devices in the child's possession. We added questions concerning video game use that we then categorized based on gaming habits. Although there was no consensus concerning this classification, video games can generally be divided in the following groups: creative games, educational games, non-violent character-based games and violent character-based or non-character-based games. We also added questions to this questionnaire for parents about their own video gaming habits and their perceptions concerning their children's gaming habits with a view to collecting preliminary data to guide our subsequent research questions (14).

Psychometric Data

QUATTORD (Questionnaire de l'attention et de l'ordinateur)

This is a questionnaire to evaluate ADHD symptoms and video game use. It includes 19 questions on ADHD symptoms (a 4-point Likert scale on which 0 = not at all and 3 = very much), nine questions on screen use (descriptive) and 11 questions on video game addiction symptoms (4-point Likert scale on which 0 = never and 3 = always). The questions on ADHD symptoms and addiction symptoms are based on the diagnostic criteria from the DSM-5 (9), and this questionnaire is easy to complete. It was used by Paulus in a study on ADHD and video gaming among more than 1,000 school and preschool-aged children and has been used since the launch of that multinational study (15). The results obtained using this questionnaire were used to generate empirical data aligned to the data in the literature.

SDQ (Strengths and Difficulties Questionnaire)

The SDQ is a validated questionnaire consisting of 25 items evaluating strengths (e.g., prosocial behaviors) and difficulties (e.g., hyperactivity, emotional regulation difficulties) in children aged 4–16 (16). We used the version to be completed by parents (there is also a version for teenagers and one to be completed by teachers). The 25 items are divided into five categories (emotional symptoms, conduct problems, attention deficit/hyperactivity, peer problems, and prosocial behaviors), each with five questions. The original, validated English version of this instrument has sound psychometric properties, which are found in the French version. Cronbach's alpha for the total difficulties score was acceptable at 0.77 (17). The goal was to use an additional independent assessment (independent from the QUATTORD) to obtain an overview of the children's general internalizing and externalizing behaviors.

Ethical Approvals

The research project was approved by the research ethics board of the CHU Sainte-Justine (the main project center). Confidentiality and anonymity were maintained throughout the study and during data transcription. The participants' identities

were coded using the numbers indicated on the envelopes. The questionnaire results were organized in an Excel spreadsheet and identified using these numbers. The computer holding this data is stored under lock and key in the research offices of the recruitment centers. If parents agreed voluntarily to participate after explanations were provided, they could then sign the consent form. Consent forms were signed in duplicate with one copy given to the parents. We also asked the children for their consent.

Statistical Analysis Strategy

First, ANOVA (analysis of variance) were conducted to compare video game playtime and addiction scores according to QUATTORD between the three study groups and between age categories for each group (4–6, 6–8, 8–10, and 10–12 years). We then clarified the differences identified by *post-hoc* analyses (Hochberg and Games-Howell). Correlation analyses were then used to assess associations between the severity of ADHD symptoms and SDQ items in relation to dependency. We then conducted a multiple regression analysis to explore the combination between the gender and having ADHD or a clinical diagnosis.

Lastly, we used multiple linear regression to evaluate associations between playtime and addiction score (continuous dependent variables obtained *via* the QUATTORD) and income, parental video gaming habits and parental perceptions concerning the impact of video gaming on their children (categorical independent variables obtained *via* the sociodemographic questionnaire).

The statistical significance threshold was $\alpha = 0.05$.

RESULTS

Descriptive Analyses

A total of 280 participants completed the study: 98 participants (35.0%) in the ADHD Group, 37 participants (13.2%) in the Clinical-Control Group and 145 in the Community-Control Group (51.8%) (**Table 1**). Participants were exclusively French-Canadian Caucasians. The mean age was 7.68 years and there was no significant difference between the three groups with respect to age ($p > 0.005$). With regard to gender, the proportion of boys and girls in the study for each group is also presented in **Table 1**.

Modalities of Use Between ADHD and Non-ADHD Children

ANOVA showed that the video game playtime was significantly higher for the ADHD Group compared to the Community-Control Group during both weekdays and weekends. The Hochberg and Games-Howell *post-hoc* analysis between the three groups were significant and identical with regard to video game playtime on weekdays and weekends. They indicated a longer time spent on video games in the ADHD Group ($p = 0.002$ for ANOVA and *post-hoc* analysis) (**Table 2**).

We compared video game addiction scores obtained at the QUATTORD by ANOVA. Addiction was significantly higher for the ADHD Group compared to the Community-Control Group ($p = 0.000$ for ANOVA and *post-hoc* analysis) (**Table 3**).

For all groups, we compared the time spent on video games between age categories by ANOVA. Since differences were only significant in the ADHD Group, we then conducted a *post-hoc* analysis in this group only. We found that time spent on video games was significantly higher for the 10–12 age group compared to the 4–6 age group during weekdays and weekends (**Table 4**).

Associations Between the Severity of ADHD Symptoms and Video Game Addiction

There was a significant correlation (Pearson correlation) for each ADHD symptom (impulsivity, inattention, hyperactivity) and video game addiction. The strongest correlation was with impulsivity (**Table 5**).

There was a significant correlation ($r = 0.182$ for p-value $= 0.003$) between hyperactivity symptoms and weekend use time. There was no significant correlation for the other symptoms (inattention and impulsivity) and the time of use during the weekend. Similarly, we did not find a positive correlation for

TABLE 1 | Proportion of boys and girl in each group.

Gender	Total	ADHD group	Clinic-control group	Community-control group
Boys	183 (65.4%)	79 (80.6%)	31 (83.8%)	73 (50.3%)
Girls	97 (34.6%)	19 (19.4%)	6 (16.2%)	72 (49.7%)
Total	280	98 (35.0%)	37 (13.2%)	145 (51.8%)

TABLE 2 | Playtime during the week and the weekend.

Group	Mean number of hours during the week	Mean number of hours during the weekend	*p*-value with respect to Community-Control Group
ADHD group	2.05	3.01	0.002 for week and weekend
Clinical-control group	1.86	2.93	0.335 for week and 0.135 for weekend
Community-control group	1.44	2.36	NA

Statistically significant $p \leq 0.05$.

TABLE 3 | Addiction scores for video games.

Group	Addiction score	*p*-value with respect to community-control group
ADHD group	1.1025	0.000
Clinical-control group	0.9355	0.119
Community-control group	0.6802	NA

Statistically significant $p \leq 0.05$.

TABLE 4 | Usage during the week and the weekend among age categories of the ADHD group.

Age category	Mean number of hours during the week	Mean number of hours during the weekend	*p*-value with respect to the 10–12 years-old age category
4–6 years-old	1.54	2.32	0.005 for the week and 0.000 for the weekend
7–9 years-old	1.95	2.95	0.027
10–12 years-old	2.69	3.79	NA

Statistically significant $p \leq 0.05$.

TABLE 5 | Correlation between symptoms of inattention, hyperactivity, impulsivity, and video game addiction.

Symptoms	Video game addiction	*p*-value
Inattention	0.279	0.000
Hyperactivity	0.294	0.000
Impulsivity	0.310	0.000

Statistically significant $p \leq 0.05$.

ADHD symptoms and the time of use of video games during the week.

Relationship Between SDQ Items and the Use of Video Games

All correlations between SDQ items and video game addiction scores found by the QUATTORD are highly significant with $p < 0.01$: positive correlations with emotional problems (0.321), conduct disorders (0.293), hyperactivity (0.242) and peer problems (0.201); and negative correlation with prosocial abilities (−0.272).

Correlations between SDQ items and playtime during the week are positive and significant for hyperactivity (0.151) and peer problems (0.143) as well as emotional problems (0.208, highly significant for this last correlation with $p < 0.01$). Correlations between SDQ items and playtime during the weekend are positive and highly significant ($p < 0.01$) for emotional problems (0.242), conduct disorders (0.164), and hyperactivity (0.229).

Exploratory Results

According to our regression analyses where we looked for variables predicting addiction, we found a significant interaction between addiction, clinical groups and gender, based on a linear regression (0.533). That is, the boys in the study showed more dependence if they belonged to one of the two clinical groups (ADHD Group and Clinical-Control Group) and, more specifically, ADHD boys had the highest addiction scores. We did not find this trend for girls with equivalent addiction scores, regardless of the group (Clinical-Control Group or Community-Control Group).

TABLE 6 | Types of video games played most frequently.

Game type	Frequency played			
	Never	Rarely	Sometimes	Often
Creative	19.2%	29.2%	26.8%	24.8%
Educational/concentration	14.9%	21.3%	37.3%	26.5
Violent human-like	62.9%	13.1%	15.5%	8.4%
Violent cartoon-based	51.2%	19.4%	19.4%	17.5%
Non-violent character-based	22.9%	17.3%	35.3%	24.5%

With respect to the types of games played, 62 participants (24.8%) often played creative games (e.g., Minecraft), 66 participants (26.5%) often played educational games (e.g., Oregon Trail), and 51 participants (8.4%) often played violent games (e.g., Call of Duty) (**Table 6**).

With respect to parents, we found that only 17.4% of parents played video games. A negative association was then identified between average household income with addiction score and children's playtime, indicating that video game use (playtime and addiction) varies inversely with income. This corresponds to the data from the scientific literature indicating that a low socioeconomic stratum is associated with development of video gaming addiction and increased playtime (18). Using linear regression, we then identified positive correlations between video game use among children (playtime and addiction score) and parental perception of the negative impact of video gaming on their children (excessive playtime and conduct problems after playing video games) (**Table 7**).

DISCUSSION

Research on the relationship between ADHD and video game addiction has mainly been conducted among adolescents and young adults, and studies like ours that focus on children are rare (19). Our study highlighted a vulnerability in ADHD children as they exhibit more addictive behaviors with respect to video games and demonstrate prolonged periods of use. We also observed a correlation between the severity of ADHD symptoms and excessive use of video games. Finally, our results suggest that the association between males and ADHD is an additional risk factor for the excessive use of video games.

Modalities of Use and Comparison Between ADHD and Non-ADHD Children

The duration of use was significantly higher in the ADHD Group compared to the Community-Control Group, during both the week and the weekend. We also found that, in accordance with the literature, when ADHD symptoms are more severe, playtime would be significantly longer (20). These results indicate that caution must be exercised when children with ADHD play video games.

TABLE 7 | Multivariate linear regression of income and parental perception with children's playtime and addiction score.

	Playtime during week (hours)		Playtime on weekends (hours)		Addiction score	
	B	p	B	P	B	p
Household income (1–6)	−11.9	0.000	−17.3	0.002	−0.9	0.051
Do you find that your child spends too much time playing video games? (No = 0; Yes = 1)	28.3	0.008	53.4	0.000	4.5	0.000
Do you find that this has positive or negative impact on your child's behavior? (Neutral/negative = 0; Positive = 1)	38	0.009	50.4	0.003	2.8	0.019

Furthermore, the degree of video game addiction was also significantly higher in children with ADHD compared to children in the community group. In the former, we indeed found, as early as preschool age, a higher tendency to develop addictive behaviors toward video games compared to children of the same age without ADHD. This would be even higher in ADHD children with behavioral problems since they might be less able to accept a certain control over their playtime. In addition, ADHD patients are at risk of addictive behavior and, more specifically, an ADHD diagnosis should increase the risk of being dependent on online video games (21).

The explanatory mechanisms of this attraction by ADHD patients to video games include becoming bored quickly, intolerance to waiting, difficulties with self-control, difficulties being motivated, the need for intense stimulation and difficulties in interpersonal relationships (22). On the other hand, studies in neurobiology have shown a release of striatal dopamine involving the brain reward circuits during video game use improving the ability to concentrate during playtime which would provide a sense of comfort for young people with ADHD (23, 24). Risk factors for the development of addiction to video games are also typical traits of ADHD (impulsivity, difficulty in managing emotions and lack of prosocial capacity, for example) (25). Video games also seem to allow young ADHD patients to offset the frustrations and failures of real life with the successes and achievements they perceive while playing, which largely explains their appeal (22).

Comparison of Usage by Age

The only significant difference found was in the ADHD Group for the 10–12 age category compared to the 4–6 age category with a longer playtime among 10–12 year-olds during the week and the weekend. According to Lemmens, the younger the video games are played, the higher the risk of developing addiction during adolescence, a period of vulnerability to addictive behavior (26). Indeed, early and regular exposure to video games with long playing sessions is one of the most common risk factors for cyberaddiction (27). According to our results, this risk factor is even more present in the ADHD Group, which presents at its core vulnerabilities to addiction.

ADHD children seem to show an increase in video game playtime as they get older, while the other two groups of children do not differ by age. This may mean that early exposure by a young ADHD patient is a risk factor for increasing use as they get older, especially at the onset of puberty and during adolescence. Indeed, adolescence is a high-risk period for addictions among young ADHD patients (12).

Associations Between ADHD Symptoms and Video Game Addiction

We observed a significant correlation between all ADHD symptoms and those of video game addiction. According to Yen, ADHD symptoms (inattention, hyperactivity and impulsivity) among ADHD patients who are also cyberaddicts would indeed appear with more intensity than in ADHD patients who are not. More specifically, inattention seems to be the most aggravated symptom of video game abuse (28). However, in our study, impulsivity appears to be the most correlated to video game addiction. This is consistent with Bioulac's hypothesis that ADHD children would also have more behavioral problems with impulsivity related to video games (29). In the same vein and with regard to the impact of screen use, the more often young people consult their smartphone, the greater the risk of developing ADHD symptoms (30).

Thus, there appears to be a link between ADHD symptoms and the excessive use of video games, although at this stage it is not possible to predict the direction of causation. Moreover, it is important to differentiate the ADHD symptoms that result from an excessive use of video games from ADHD as a neurodevelopmental disorder.

Association Between SDQ Items and the Use of Video Games

We found a highly significant positive association between behavioral problems on one hand, and video game addiction and playtime during the weekend on the other. The uncontrolled use of screens during childhood, whether for video gaming or otherwise, would therefore lead to a high risk of behavioral difficulties. Similarly, according to a study by Poulain et al. among children aged 2–6, the use of screens (including video games) is a major risk factor for the development of behavioral difficulties (31). Moreover, there is a strong association between early exposure to screens and the subsequent development of aggressive and antisocial behaviors (32, 33). In light of all those results and ours, it appears that excessive use of video games may

negatively influence emotional and behavioral problems and the well-being of children from their early years.

We obtained a positive and significant correlation between socialization difficulties and video game addiction and playtime during the week, while the association between video game addiction and prosocial abilities was negative. Indeed, according to the literature, there is a strong association between increased screen time and reduced social development in children (34). Socialization difficulties are risk factors for video game addiction (27) and at the same time, they will be encouraged by the use of screens by pushing young people to overuse them in avoidance behaviors. Among young people who are not socially at ease and experience a sense of failure in their lives, online interactions reduce negative feelings such as loneliness and boredom (6). That being said, when parents limit and monitor screen time, children would develop better prosocial skills.

Exploratory Results

Genre

We looked for the implication of gender and more specification for the use of video games. First, a majority of boys were in both clinical groups (ADHD Group and Clinical-Control Group). This corresponds to the clinical and epidemiological reality where the gender ratio for ADHD is two boys for one girl and where other neurodevelopmental disorders are also predominantly found in males. Moreover, according to Paulus et al., more boys than girls have video game consoles, and more girls than boys are non-players (11). We looked for covariables that influence addiction through a regression analysis. According to this regression, if we look at the interactions between groups and gender, ADHD boys appear to be the most at risk of video game addiction compared to boys in the other two groups. On the other hand, there does not seem to be any differences with respect to girls. Boys with a child psychiatry diagnosis of ADHD, therefore, seem to be the most vulnerable to video game addiction. It would then be a question of orienting the evaluation and care according to this fragility, which should not be neglected.

Video Games

It should be noted that studies on video games do not usually take into account the type of video games played, yet it seems essential to begin to make such distinctions if we want to make accurate recommendations. There are indeed many different types of video games and different ways of playing that likely have an impact on potential overuse. In this preliminary study, we tried to describe the games played by the children in order to get an initial overview of the situation. We found that the most popular video games played among 4–12 year-olds were educational rather than violent games. Educational games (those designed for a primary purpose other than pure entertainment) have pedagogical virtues that can be particularly useful for children with learning difficulties (35). This type of data can be used to refine future research and analysis in order not only to prevent the negative aspects of video games, but also to optimize their use.

The data from the literature also suggest that the relationship between ADHD symptom severity and video game addiction severity depends on the type of game preferred or played most often. This relationship may also depend on the level of reinforcement of the most-played game; that said, the reinforcement strategies of video games are constantly evolving to attract more and more players and become as addictive as possible. These changes have been bolstered by the initial success of massively multiplayer online role-playing games (MMORPGs), which have strong reinforcement structures, followed by other games that have increased reinforcement levels while creating a potentially higher risk of developing excessive use (e.g., Fortnite).

Parental Impact

No data exists regarding the use of video games by parents. In our study, we found that a majority of parents do not play video games at all. This has an impact on the parents' understanding and management of video games. Ideally, it is recommended that screen time be shared between parents and children according to the Canadian Pediatric Society guidelines (36). Poor relationships with parents, poor parental control, hostile parenting, and lack of rules on screen use are risk factors for video game addition. Parents must serve as role models with regard to screen use (37).

We identified a negative association between income with addiction score and playtime, indicating that video game use (playtime and addiction) varies inversely with income. This corresponds to the data from the scientific literature indicating that a low socioeconomic stratum is associated with development of video gaming addiction and increased playtime (38). Radesky and Christakis hypothesizes that parents from lower socioeconomic strata have fewer educational requirements in relation to video game use. Additionally, these parents appear to use video games as a pastime more frequently (39). Our results reflected this observation.

We explored video game use by parents, since the literature was weak in this area (37). Parental modeling and educational guidelines for video gaming appear to have an impact on IGD. The Canadian Pediatric Society (CPS) also recommends that parents use screen time to create opportunities for shared activities with children, and a majority of parents in our study did not play video games (37). We also identified a positive correlation between video gaming among children (playtime and addiction score) and parental perception of excessive playtime among their children. When it comes to estimating their children's playtime, parents appear to tend to overestimate the time their children dedicate to socially desirable activities (e.g., reading, homework) and underestimate the time they spend on socially undesirable activities (e.g., television, video games) (40). Moreover, many parents find that the official recommendations concerning children's screen time from pediatric learned societies such as the American Academy of Pediatrics (AAP) or the CPS are not realistic and could never be put into practice (41). They assert that they do not know how much screen time or, in particular, video game playtime is healthy or excessive (42). Parental evaluation of screen time in our study questionnaires may be erroneous if parents are confused about the recommendations or biased by social desirability.

We found a positive correlation between video game use in children (playtime and addiction score) and parental perception of negative impact on their children's behavior after playing video games. To our knowledge, the links between video game use among children and this negative perception have never been discussed. As part of this exploratory analysis, we were consequently able to collect preliminary data on this topic, and it may be interesting to look at other possible correlations based on the data collected through our questionnaires. It is important to note that these results apply only to a clinical population (clinical and community groups). Although parental concerns are underscored, they may also be somewhat directed in that parents completing the scales for the project could be particularly concerned about this issue in relation to their children. According to Schneider et al., a positive perception of video gaming could lead to more frequent usage problems in children (43), whereas others have indicated that a positive perception does not have any impact. Regardless, the concept of perception remains highly subjective, multifaceted and difficult to capture.

Time of Year of Questionnaire Completion

We wanted to include this information, as it could create a bias in relation to data collection concerning video game use in that the majority of questionnaires were completed during the summer, when, based on our observations in the clinical setting, in the context of vacation and the absence of a structured schedule, children very likely spend more time playing video games. Recruitment levels were also higher due to the availability of research students.

STRENGTHS AND LIMITATIONS

Strengths

Although certain limitations and strengths were identified, we wish to take the reflection process further. The study has a certain number of strong points, such as the facts that the population was made up of children at least 4 years of age in the literature on video gaming and that the study was multicentre to ensure that the population was as varied as possible, thereby corresponding more closely to the general population. Our study is also very interesting in that it included control groups, the use of which enhances validity, particularly since the study involved evaluating behaviors in a specific segment of the population. The comparisons in the study are all the more relevant and useful since, in addition to the community group, we had a clinical control group, making even more detailed comparisons possible. From a statistical viewpoint, we took care to perform, with support from statisticians, appropriate analyses for our variables taking our hypotheses into account. We were successful in fully characterizing the population and the data on parents. Lastly, we were able to establish a highly comprehensive overview of the current knowledge based on a review of the literature.

We used multiple validated, reliable, standardized measures; a wide variety of measurement scales on video game addiction have been used in research on that topic, but none has been validated. Additionally, numerous studies have used non-representative, self-selected samples and/or small samples. This both compromises the comparability of results and raises questions as to diagnosis validity in participants. In this regard, the lack of consensus and use of overly broad criteria imply the overly broad use of the diagnosis of video game addiction in cases that could instead be classified as excessive behaviors not resulting in functional impairment and falling within the societal reality of screen use.

Similarly, in contrast to the majority of ADHD studies, in which the diagnosis is not confirmed, the ADHD diagnosis of our participants was made following comprehensive assessment at specialized clinics. The same applies to diagnosis in the clinical control group. What is more, the use of multiple comprehensive questionnaires further supports the validity of our results.

Limitations

The results of this study should be interpreted in light of its limitations. Since the questionnaires are completed by the parents themselves, there may be a measurement bias with regard to their subjectivity. In addition, the possibility of an ADHD diagnosis in the community population has not been eliminated and could lead to a bias in the results. Lastly, it must be noted that the study is not longitudinal, and we were also not able to test any mechanisms that could explain patterns of findings. Additionally, questions are asked only about video game use at T0 of questionnaire completion, which does not allow for a broader perspective over time. Playtime may also be underestimated, since the measures are based on scales completed by the parents, who may have been influenced by lack of knowledge about actual playtime and/or social desirability to indicate values lower than actual. The possibility of ADHD diagnosis in the community population was not ruled out and could lead to a bias in the results.

Perspectives and Reflection

The data collected on video gaming and parents are exploratory. These topics are of interest, and based on these initial results, we can now delve further into our hypotheses and direct our research questions. Next, diagnoses in the clinical control group were documented but not analyzed and could be incorporated into future subanalysis efforts to explore the impact of other diagnoses in child psychiatry (also by expanding recruitment in this subgroup). In addition, the questionnaires were completed by only one of two parents, which could lead to different responses if parents were separated or had different approaches to managing screen time (which we see frequently in the clinical setting). However, in our characterization of the population, we noted that a large majority of parents in our sample were not separated. We also did not track refusals, and the parents who consented appeared very willing to assist, which could indicate underlying problems (at least in their view).

This project lacks a longitudinal aspect, which is conspicuously absent from the literature. To create the most accurate portrait possible of video game use, this activity would have to be tracked over time, while also avoiding situational use. For example, we observed that the majority of recruitment took place in the summer, whereas use doubtless varies depending

on whether school is in session or children have a structured schedule as well as their options for outdoor activities.

The current COVID-19 pandemic has greatly affected lifestyles and screen time. Already omnipresent in our lives, screens have become the only means of working, entertaining ourselves, keeping in touch with loved ones, socializing and learning. Moreover, screens became a go-to solution for keeping children busy while parents continued working from home and daycare facilities and schools remained closed. With regard specifically to video gaming during the pandemic, video game use increased significantly during lockdown periods (also generating exponential earnings growth over a period of a few months for the video game industry), but this greater use could also be only temporary.

A next step would be to undertake analyses and explore the impact of video games on family dynamics and academic performance, document the types of video games used and identify any comorbidities potentially influencing this use. Then, although we have studied the adverse effects of video gaming in terms of addiction, it would be useful nonetheless to examine any benefits it may also have. For example, video games may also help to develop certain skills (sense of control, socialization, coordination, etc.) (44) and offer new educational and therapeutic possibilities.

Knowledge about video game addiction continues to grow, and we continue to learn more about potential vulnerability to this addiction among certain individuals based on their mental health diagnosis. In particular, an ADHD diagnosis appears to be a major factor in the development of a video gaming addiction, although this association also appears bidirectional and is not yet fully understood. At the same time, studies on the relationship between ADHD and video game addiction have been conducted primarily in teenagers and young adults and have rarely targeted children. Our study consequently underscores the vulnerability of children with ADHD to excessive video gaming and the consequences of gaming on their symptomatology. We wanted to supplement the analysis component of our study by exploring the role of internal (age, sex) and external (socioeconomic stratum, parents' role) factors, thereby corroborating the concept of a multifaceted relationship behind addictive video gaming behaviors.

CONCLUSION

ADHD symptoms and video game addiction appear to have a bidirectional relationship in which the ADHD symptoms make video gaming appealing, while play itself exacerbates the ADHD symptoms by providing an activity that continually reinforces the need for instant gratification. Long hours of video gaming may further reinforce and consolidate children's propensity to uncontrolled reactivity and pervasive impatience without coaching to develop more reflection-oriented behaviors. As stated previously, the time dedicated to video gaming is spent at the expense of leisure activities such as sports, music and the arts, which would assist in developing children's attention, self-assurance, behavioral inhibition, discipline, team skills, and socialization. In this context, related studies provide opportunities to reflect on the potential impact on our practice. By exploring the consequences of intensive video gaming for these young children, it is possible to develop intervention approaches to propose to professionals.

Although we understand that video games present risks, it would still be interesting to look at the benefits they can bring. Indeed, it is important to note that they can also help in developing skills such as a sense of control and coordination (45). There is a definite interest in using them as a lever for young people by offering new educational and therapeutic perspectives.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Centre de recherche du CHU Sainte-Justine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Role of mTOR-Regulated Autophagy in Synaptic Plasticity Related Proteins Downregulation and the Reference Memory Deficits Induced by Anesthesia/Surgery in Aged Mice

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Postoperative cognitive dysfunction increases mortality and morbidity in perioperative patients and has become a major concern for patients and caregivers. Previous studies demonstrated that synaptic plasticity is closely related to cognitive function, anesthesia and surgery inhibit synaptic function. In central nervous system, autophagy is vital to synaptic plasticity, homeostasis of synapticproteins, synapse elimination, spine pruning, proper axon guidance, and when dysregulated, is associated with behavioral and memory functions disorders. The mammalian target of rapamycin (mTOR) negatively regulates the process of autophagy. This study aimed to explore whether rapamycin can ameliorate anesthesia/surgery-induced cognitive deficits by inhibiting mTOR, activating autophagy and rising synaptic plasticity-related proteins in the hippocampus. Aged C57BL/6J mice were used to establish POCD models with exploratory laparotomy under isoflurane anesthesia. The Morris Water Maze (MWM) was used to measure reference memory after anesthesia and surgery. The levels of mTOR phosphorylation (p-mTOR), Beclin-1 and LC3-II were examined on postoperative days 1, 3 and 7 by western blotting. The levels of synaptophysin (SYN) and postsynaptic density protein 95 (PSD-95) in the hippocampus were also examined by western blotting. Here we showed that anesthesia/surgery impaired reference memory and induced the activation of mTOR, decreased the expression of autophagy-related proteins such as Beclin-1 and LC3-II. A corresponding decline in the expression of neuronal/synaptic, plasticity-related proteins such as SYN and PSD-95 was also observed. Pretreating mice with rapamycin inhibited the activation of mTOR and restored autophagy function, also increased the expression of SYN and PSD-95. Furthermore, anesthesia/surgery-induced learning and memory deficits were also reversed by rapamycin pretreatment. In conclusion, anesthesia/surgery induced mTOR hyperactivation and autophagy impairments, and then reduced the levels of SYN and PSD-95 in the hippocampus.

An mTOR inhibitor, rapamycin, ameliorated anesthesia/surgery-related cognitive impairments by inhibiting the mTOR activity, inducing activation of autophagy, enhancing SYN and PSD-95 expression.

Keywords: rapamycin, mTOR, postoperative cognitive dysfunction, aged, synaptic plasticity

INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a complication of the central nervous system after anesthesia and surgery, mainly manifested in the deterioration of cognitive function, especially learning and memory, attention (Abildstrom et al., 2000; Berger et al., 2015; Steinmetz and Rasmussen, 2016; Holmgaard et al., 2019). It may last for days, months or even years, result in the increase of hospitalization expense and extension of hospitalization time, and markedly impair postoperative recovery and increase morbidity and mortality (Steinmetz et al., 2009; Bilotta et al., 2010; Quan et al., 2019). Although POCD is an important clinical issue, the pathogenesis of POCD remains to be fully elucidated. Furthermore, clarifying its pathogenesis can help to prevent its occurrence.

Mammalian target of rapamycin (mTOR) is a central regulator of protein synthesis in neurons. Several studies have revealed that mTOR signaling may be correlated to neurodegenerative diseases (Garelick and Kennedy, 2011; Heras-Sandoval et al., 2014), including Parkinson disease, Huntington's disease, Alzheimer's disease (Bockaert and Marin, 2015). In Alzheimer's disease, the inhibition of mTOR by rapamycin ameliorates the synthesis of β-amyloid and tau protein and improves learning and memory abilities (Spilman et al., 2010). mTOR is necessary for phagophore and autophagosome formation. In the central nervous system, autophagy and its regulation by mTOR (Costa-Mattioli and Monteggia, 2013) are critical for maintaining neuronal functions (Kulkarni and Maday, 2018), such as synaptic remodeling and plasticity associated with long-term memory formation (Shehata and Inokuchi, 2014). Montesinos et al. (2018) have demonstrated that autophagy is impaired by increasing autophagy inhibitor mTOR. Inhibition of mTOR, by rapamycin, reestablishes the LC3-II levels and restores the levels of synaptic plasticity-related proteins including PSD-95, SHANK3 and p62. These suggest that mTOR-regulated autophagy system plays an important role in the synaptic plasticity-related proteins alterations. SYN and PSD-95 are synaptic functional proteins, which are related to synaptic plasticity (Pan et al., 2017; Liu et al., 2018; Ma et al., 2019). Therefore, in the present study, we explored that whether the modulation of the autophagy activation, by inhibiting mTOR with rapamycin administration, was capable of restoring alterations in the decline of synaptic plasticity-related proteins and the cognitive impairment induced by anesthesia/surgery.

MATERIALS AND METHODS

Ethical approval for this study was provided by the Animal Care and Use Committee of the Second Affiliated Hospital of Jiaxing University. All animal procedures were performed in accordance with the National Institutes of Health animal care guidelines.

Animals, Anesthesia/Surgery and Procedure Pharmacological Treatment

Anesthesia was prepared using C57BL/6Jmice (18-month old, male) were provided by Hunan SJA Laboratory Animal Company, Limited. The animals were housed under controlled conditions (temperature of 22 ± 2°C, 12 h light/12 h dark cycle) with access to food and water *ad libitum*. All mice were allowed to adapt to their new environment for 7 days before beginning the experiments.

The mice were randomly divided into four groups: control group (mice received injections of vehicle without anesthesia/surgery), control+rapa group (mice received rapamycin pretreatment without anesthesia/surgery), anesthesia/surgery group (mice received anesthesia/surgery with injections of vehicle) and anesthesia/surgery+rapa group (mice received anesthesia/surgery with rapamycin pretreatment). Exploratory laparotomy was performed under isoflurane anesthesia (induced with 4.0% isoflurane and maintained with 2.0% isoflurane in 0.30 FiO_2). First, a midline laparotomy (3 cm in length) was performed. Second, the sterile cotton swabs were dipped in the wet normal saline, and the abdominal organs were explored successively. The exploration in the abdominal cavity lasted for 3 min and the exposure lasted for 3 min. Aseptic techniques were used during the entire procedure, which required approximately 30 min. Third, around the incision, 0.1% lidocaine infiltration was used as postoperative analgesia, and then the wound was closed by 5–0 Vicryl sutures. The wound was covered with bacitracin ointment. The mice were gently restrained to a heating pad (37°C) using paper tape, and the whole procedure lasted 20 min. The dose of rapamycin used was selected based on previous studies (Tang et al., 2014). The mice received intraperitoneal injections of 0.5 mg/kg/day rapamycin for 3 days prior to undergoing exploratory laparotomy.

We used the Morris Water Maze (MWM) to test the reference memory of aged mice. Mice were euthanized on days 1, 3 and 7 days after anesthesia/surgery in each group. Hippocampus tissue was dissected for western blot analysis, and stored at −80°C.

Morris Water Maze (MWM)

The MWM test was conducted in a large circular pool (110 cm in diameter and 30 cm in depth) made of white plastic. The pool was filled with water to a depth of 35 cm (maintained at 23–25°C), covering a transparent circular platform 10 cm in diameter. The platform was submerged approximately 1.0 cm below the surface of the water. The pool was situated in a room with diffuse lighting and was surrounded by curtains. The curtains were white and had

distinct cues painted on them. The position of the cues remained unchanged throughout the task. The swimming activity of each mouse was monitored and tracked *via* a television camera (HIK VISION Company, Limited, Hangzhou, China) mounted overhead, which relayed information, including variables such as latency to the platform, total distance traveled and time spent in each quadrant, to a video-tracking system (RWD Company, Limited, Shenzhen, China).

After the anesthesia/surgical procedure, training was conducted for 4 days for the reference memory test. Each day, a trial was initiated by placing each mouse in the water facing the pool wall in one of the four quadrants, which was randomly selected. For each training trial, the mouse was allowed to swim for 60 s to find the hidden platform. When successful, the mouse was allowed a 30-s rest period on the platform. If the mouse failed to find the platform within 60 s, it was gently guided to the platform and allowed a 30-s rest period on the platform. On the fourth day of training, the latency of each group was analyzed. The probe trials were conducted after the training trials. In this test, the platform was removed from the pool and the mice were allowed to search for it for 60 s. The time spent in the target quadrant, where the platform is placed, was analyzed.

Western Blotting

The hippocampal tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitors, and then centrifuged at 4°C at 12,000 *g* for 20 min. The concentration of protein in the supernatants was determined using a Bradford protein assay kit (Servicebiotechnology Company, Limited, Wuhan, China). Equal quantities of the protein samples were denatured at 100°C for 15 min, and were then separated by10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically onto a polyvinylidene fluoride membrane (Millipore). The membranes were blocked using 5%-TBST for 60 min and then incubated with the following primary antibodies: mTOR phosphoS2448 (p-mTOR, 1:1,000; Abcam), postsynaptic density protein 95 (PSD-95, 1:1,000; Abcam) and synaptophysin (SYN, 1:1,000; Abcam) at 4°C overnight. Following the membranes were washed three times (5 min each) in TBST and incubated with secondary antibody for 60 min at room temperature. The membranes were detected using an enhanced chemiluminescence system (CLINX, China) and analyzed using AlphaEaseFC (Alpha Innotech).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR was performed on the ABI7900 (Illumina) with FastStart Universal SYBR Green Master(Rox) (Roche). cDNA was synthesized from RNA (65°C for 5 min, 42°C for 60 min, and 70°C for 5 min) using RevertAid First Strand cDNA Synthesis Kit (Thermo). The DNA was amplified (95°C for 10 min; and 40 cycles of 95°C for 15 s, 60°C for 60 s). The sequences of specific primers are summarized in **Table 1**. The expression level of each gene was calculated based on the comparative threshold cycle value (Ct) method, using the formula $2 - \Delta\Delta Ct$ ($\Delta\Delta Ct = \Delta Ct$ sample $- \Delta Ct$ reference). The final results were expressed as normalized fold values relative to the control group.

Statistical Analysis

Data are presented as the mean ± SEM. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software Inc., USA). A two-way ANOVA was used to analyze the water maze escape latency and average speed, and relative protein levels of p-mTOR, Beclin-1 and LC3-II, SYN and PSD-95. A one-way ANOVA or unpaired *t*-test was used to analyze the probe quadrant trial data, probe test data. The data were checked for normality before running ANOVA. $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Anesthesia and Surgery Impaired Reference Memory in Aged Mice

To determine whether anesthesia/surgery produced cognitive dysfunction in aged mice, we used the MWM to evaluate learning and memory after exploratory laparotomy. As shown in **Figure 1A**, mice were acclimated to the environment for 7 days before the experiments were conducted. Exploratory laparotomy was performed under isoflurane anesthesia, the mice were allowed to rest for 2 days after the surgery. The training course for the water maze lasted for 4 days, probe tests were conducted on day 7 (**Figure 1A**). Two-way ANOVA revealed that the mean latency of the mice from the anesthesia/surgery group was significantly prolonged on the fourth day of training than the control mice ($F_{(1,88)} = 8.686$; $p < 0.01$; **Figure 1B**). Two-way ANOVA revealed that the average swimming speed during the training and probe tests were not significantly different between the two groups (**Figure 1C**), this excluded the effects of motor and perceptual abilities on spatial learning and memory after anesthesia/surgery. In the probe test, unpaired *t*-test revealed that swimming time of the anesthesia/surgery group in the target quadrant was significantly shorter than the control group ($t = 4.422$; $p < 0.001$; exploratory path of two groups of mice in the probe test; **Figure 1D**); ANOVA revealed that the time spent in the target quadrant was significantly higher than the other quadrants in control mice ($F = 19.13$; $p < 0.001$; **Figure 1E**). Anesthesia/surgery did not impair reference memory function in young mice (Zhao et al., 2016). These results suggested that anesthesia/surgery impaired reference memory in aged mice.

Anesthesia and Surgery Induced the Upregulation of p-mTOR and Decreased LC3-II and Beclin-1 Expression in the Hippocampus

To investigate the effect of anesthesia/surgery on p-mTOR, LC3-II and Beclin-1 levels in the hippocampus, the present study detected the levels of p-mTOR, LC3-II and Beclin-1 levels in the homogenates of the hippocampal tissue using western blot analysis (**Figure 2A**). Two-way ANOVA revealed that anesthesia/surgery increased the production of p-mTOR ($F_{(1,30)} = 145.9$; $p < 0.001$) and decreased

TABLE 1 | The primer sequence.

Name	Forward	Reverse	Size (bp)
GAPDH	5′-CCTCGTCCCGTAGACAAAATG-3′	5′-TGAGGTCAATGAAGGGGTCGT-3′	133
SYN	5′-GGCGGCACTTCTGTCATCAA-3′	5′-AATGACACCTCCCAGCACTTC-3′	225
PSD-95	5′-CGATTACCACTTTGTCTCCTCCC-3′	5′-ACGGATGAAGATGGCGATAGG-3′	220

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SYN, synaptophysin; PSD-95, postsynaptic density protein 95.

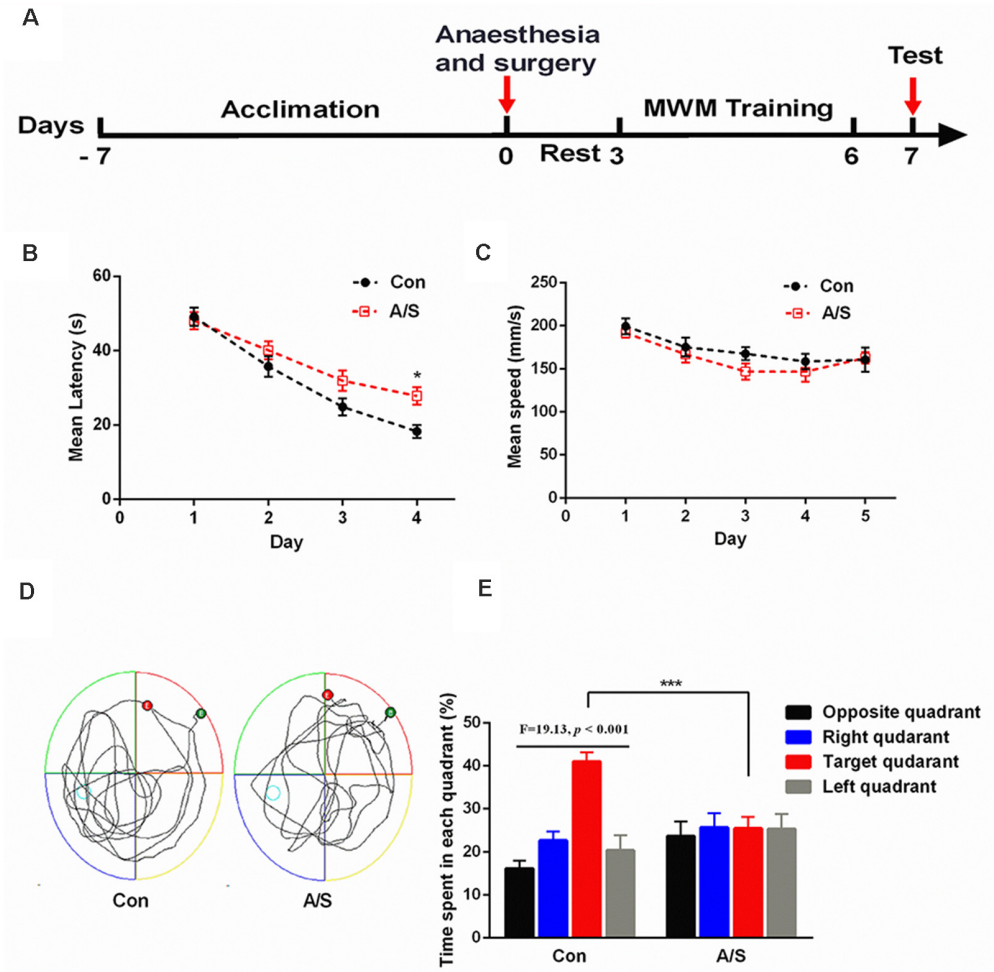

FIGURE 1 | Anesthesia and surgery impaired reference memory in aged mice. **(A)** Schematic timeline of the experimental paradigm of surgery procedure and Morris water maze test. After the anesthesia/surgical procedure, training was conducted for 4 days followed by probe tests on day 7. **(B)** Escape latency to reach the hidden platform during the 4-day training; the mean latency of the anesthesia/surgery group was significantly prolonged on day 4 than the control. **(C)** Average swimming speed; there was no significant difference between the two groups. **(D)** Representative exploratory path of two groups of mice in the probe test. **(E)** The percentage of time spent in the target quadrant during the probe test; there was significant difference between the control group and the anesthesia/surgery group. The percentage of time spent in the target quadrant was significantly higher than the other quadrants in control mice. Data are mean ± SEM (n = 12 per group). ***$p < 0.001$ vs. Con. Con, control group; A/S, anesthesia/surgery group.

LC3-II ($F_{(1,30)}$ = 414.1; $p < 0.001$) and Beclin-1 expression ($F_{(1,30)}$ = 448.8; $p < 0.001$) on days 1, 3 and 7 after operation, compared with the control group, as shown in **Figures 2B–D**. These data indicated that anesthesia/surgery increased the expression of p-mTOR and decreased the levels of LC3-II and Beclin-1 on days 1, 3 and 7 after anesthesia/surgery.

Anesthesia and Surgery Reduced SYN and PSD-95 Levels in the Hippocampus

In order to investigate the effect of anesthesia/surgery on the production of SYN and PSD-95, the present study detected the levels of SYN and PSD-95 in the homogenates of the hippocampal tissue using western blot analysis (**Figure 3A**). Two-way ANOVA revealed that anesthesia/surgery decreased

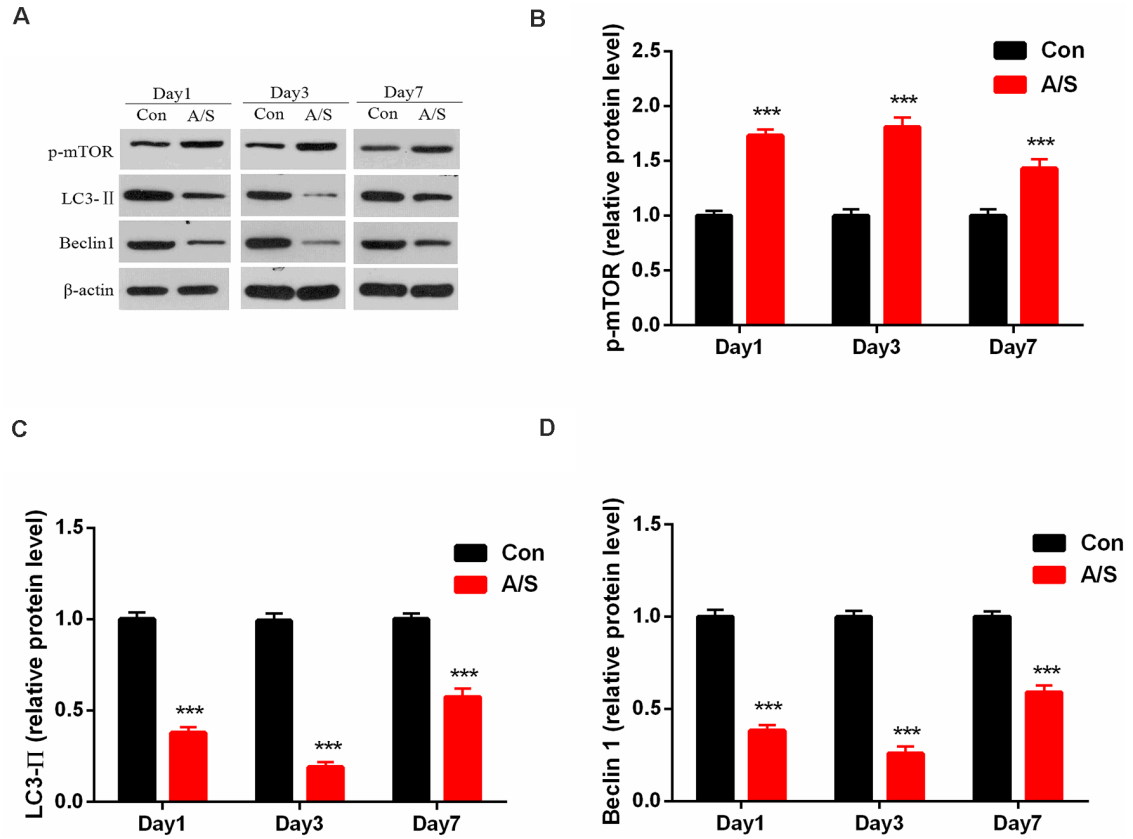

FIGURE 2 | Anesthesia and surgery increased the activation of mammalian target of rapamycin (mTOR) and decreased LC3-II and Beclin-1 levels in the hippocampus. **(A)** Representative western blot illustrating p-mTOR, LC3-II and Beclin-1 levels in the hippocampus on days 1, 3 and 7 after surgery. **(B)** Anesthesia/surgery increased p-mTOR expression in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. **(C,D)** Anesthesia/surgery reduced LC3-II and Beclin-1 levels in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. Data are mean ± SEM (n = 6 per group). ***$p < 0.001$ vs. Con. Con, control group; A/S, anesthesia/surgery group.

the production of SYN ($F_{(1,30)} = 47.78$; $p < 0.001$) and PSD-95 ($F_{(1,30)} = 85.89$; $p < 0.001$) on days 1, 3 and 7 after operation, compared with the control group, as shown in **Figures 3B,C**. These data indicated that anesthesia/surgery decreased the levels of SYN and PSD-95 on days 1, 3 and 7 after anesthesia/surgery.

Rapamycin Treatment Inhibited the Abnormal p-mTOR and Increased LC3-II and Beclin-1 Levels in the Hippocampus

In order to investigate whether the hyperactivation of p-mTOR was involved in the reduction of LC3-II and Beclin-1 levels, the present study examined the effect of rapamycin on the levels of LC3-II and Beclin-1 in the homogenates of the hippocampal tissue using western blot analysis (**Figure 4A**). It was found that rapamycin significantly increased the production of LC3-II ($F_{(3,60)} = 160.9$; $p < 0.001$) and Beclin-1 ($F_{(3,60)} = 100.4$; $p < 0.001$), and attenuated the levels of p-mTOR ($F_{(3,60)} = 85.97$; $p < 0.001$) on days 1, 3 and 7 after operation by using two-way ANOVA, compared with the anesthesia/surgery group, as show in **Figures 4B–D**. No significant difference was found for p-mTOR, LC3-II and Beclin-1 levels between Con group and Con+Rapa ($p > 0.05$; **Figure 4**). Notably, mTOR inhibition by rapamycin administration was able to restore p-mTOR increase and LC3-II and Beclin-1 reduction induced by anesthesia/surgery in aged mice.

Rapamycin Pretreatment Increased SYN and PSD-95 Levels in the Hippocampus

In order to investigate whether the hyperactivation of p-mTOR was involved in the reduction of SYN and PSD-95, the present study examined the effect of rapamycin on the levels of SYN and PSD-95 in the homogenates of the hippocampal tissue using western blot analysis (**Figure 5A**). It was found that rapamycin significantly increased the production of SYN ($F_{(3,60)} = 35.35$; $p < 0.001$) and PSD-95 ($F_{(3,60)} = 54.66$; $p < 0.001$) on days 1, 3 and 7 after operation by using two-way ANOVA, compared with the anesthesia/surgery group, as shown in **Figures 5B,C**. There was no difference in the levels of SYN and PSD-95 between Con group and Con+Rapa ($p > 0.05$; **Figure 5**). Notably, rapamycin preadministration was capable of restoring the SYN and PSD-95 levels.

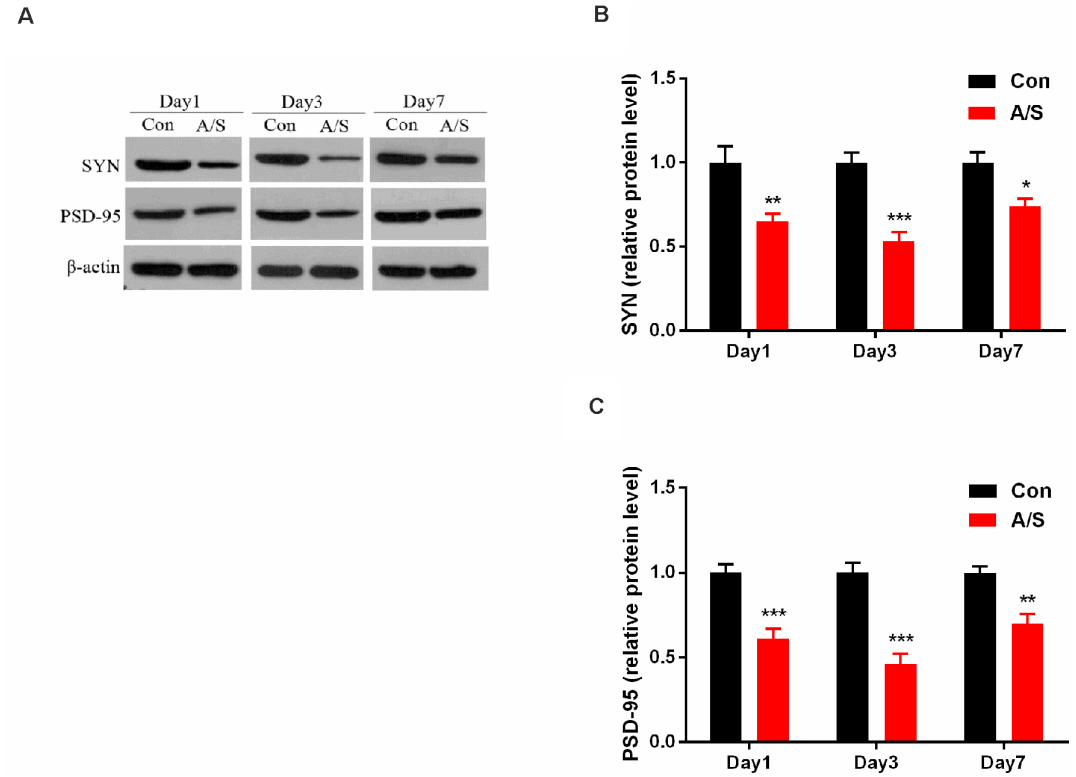

FIGURE 3 | Anesthesia and surgery decreased synaptophysin (SYN) and postsynaptic density protein 95 (PSD-95) levels in the hippocampus. **(A)** Representative western blot illustrating SYN and PSD-95 levels in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. **(B,C)** Anesthesia/surgery reduced SYN and PSD-95 expression in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. Data are mean ± SEM (n = 6 per group). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. Con. Con, control group; A/S, anesthesia/surgery group.

Anesthesia/Surgery and Treatment With Rapamycin Were Not Significantly Affected the mRNA Levels of SYN and PSD-95 in the Hippocampus

The mRNA levels of SYN and PSD-95 in the hippocampus were measured by qRT-PCR on days 1, 3 and 7 after anesthesia/surgery. No significant change was shown in mRNA levels of SYN and PSD-95 in the hippocampus at different time points after anesthesia/surgery ($p > 0.05$; **Figure 6**). These data indicated that treatment with rapamycin did not significantly increase the mRNA levels of SYN and PSD-95 in the hippocampus in aged mice.

Rapamycin Alleviated the Negative Effects of Anesthesia/Surgery on Reference Memory

Previous studies demonstrated that the increased p-mTOR contributed to the cognitive dysfunction caused by anesthesia/surgery. The effect of rapamycin, the mTOR inhibitor, administration on learning and memory function was determined using a MWM assessment, which was also used to measure hippocampal-dependent learning and memory. After 3 days treatment with rapamycin, exploratory laparotomy was performed. After 2 days of rest, we evaluated spatial reference memory with the Morris water maze (**Figure 7A**). As the training days increased, the mean latency of four groups decreased; and it was significantly higher in the anesthesia/surgery group than in the other three groups on the fourth day of the training period ($F_{(3,176)} = 5.846$, $p < 0.001$; **Figure 7B**). The average swimming speed during the training and probe tests was not significantly different among the four groups ($p > 0.05$; **Figure 7C**; exploratory path of four groups of mice in the probe test; **Figure 7D**). During the probe test, mice given rapamycin exhibited a similar preference for the target quadrant as the control group. Meanwhile, mice in the rapamycin + anesthesia/surgery group could significantly reverse the compromised preference for target quadrant; data compared with two-way ANOVA ($F_{(3,176)} = 37.04$, $p < 0.001$; **Figure 7E**). These data suggested that rapamycin treatment rescued the impairment of hippocampal-dependent memory induced by anesthesia/surgery.

DISCUSSION

In this study, anesthesia and surgery induced hyperactivation of mTOR and decreased the levels of autophagy related proteins including Beclin-1 and LC3-II in the hippocampus.

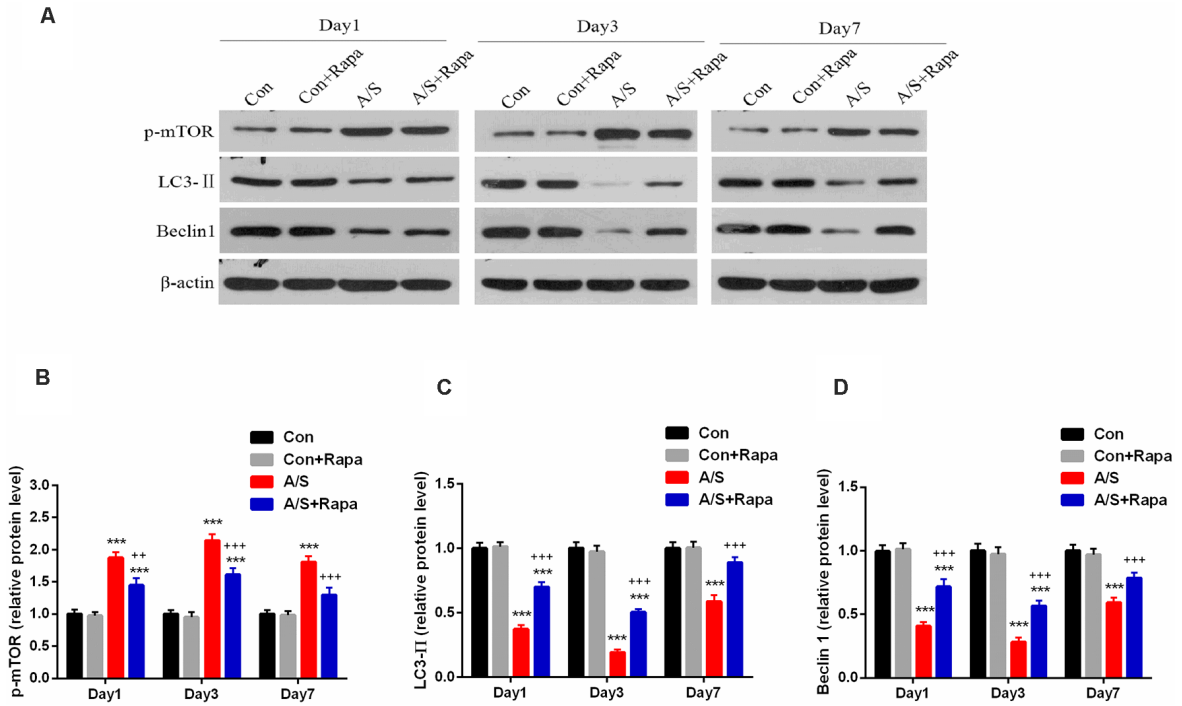

FIGURE 4 | Rapamycin treatment inhibited the abnormal p-mTOR and increased LC3-II and Beclin-1 levels in the hippocampus. **(A)** Representative western blot illustrating the effects of rapamycin on the anesthesia/surgery-induced changes in hippocampal p-mTOR, LC3-II and Beclin-1 levels on days 1, 3 and 7 after anesthesia/surgery. **(B)** Rapamycin reversed anesthesia/surgery-induced increase in p-mTOR expression in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. **(C,D)** Rapamycin reversed anesthesia/surgery-induced decrease in LC3-II and Beclin-1 levels in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. Data are mean ± SEM ($n = 6$ per group). $^{***}p < 0.001$ vs. Con. $^{+++}p < 0.001$, $^{++}p < 0.01$ A/S. Con, control group; Con+Rapa, control group with rapamycin pretreatment; A/S, anesthesia/surgery group; A/S+Rapa, anesthesia/surgery group combined with rapamycin pretreatment.

The inhibition of autophagy, reduction of neuronal/synaptic plasticity-related proteins such as SYN and PSD-95 were involved in memory and cognitive impairment. Inhibiting the mTOR hyperactivation with rapamycin administration improved anesthesia/surgery-induced memory and cognitive dysfunction by activating autophagy and increasing synaptic plasticity related proteins SYN and PSD-95 expression. Thus, this present study suggests that inhibiting excessive activity of mTOR and activating autophagy may provide a restorative method for the treatment of anesthesia/surgery-induced cognitive impairment.

The Morris water maze is a common test of cognitive function, which can objectively reflect the spatial learning and memory ability of animals. The swimming speeds during the training and probe tests were comparable between the control mice and those from the surgery group, this reduced interference to the test results. In this study, the avoidance latency period was used to judge the spatial learning ability of mice, time spent in the target quadrant was used to judge the spatial memory ability of mice. Our results showed that during the training course, the mice from the anesthesia/surgery group showed longer escape latency compared with the mice in the control group. During the probe test, the preference for the target quadrant was significantly receded in mice that had undergone anesthesia/surgery compared to control mice. These results suggested that anesthesia/surgery impaired reference memory in aged mice. mTOR inhibitor (rapamycin) pretreatment significantly compromised the decreased preference for target quadrant caused by anesthesia/surgery. During the training course, the escape latency of mice in the A/S+Rapa group was significantly higher on day 4 than the A/S group. These data suggested that anesthesia/surgery induced the hyperactivity of mTOR, which was involved in the postoperative cognitive deficits.

The mammalian target of rapamycin (mTOR) is an atypical serine/threonine kinase, and it is critical in cell growth, proliferation, protein synthesis, autophagy (Laplante and Sabatini, 2012; Efeyan et al., 2015). Autophagy is utilized by cells to clear damaged proteins which play an essential role in the neurodegenerative diseases (Scrivo et al., 2018; Djajadikerta et al., 2020). With regard to autophagy, Beclin-1 is considered as a common index for detecting autophagy. Beclin-1 plays a key role in triggering the formation of autophagosome and is essential for the recruitment of other autophagy-related proteins (Sinha et al., 2008). In the AD mice, Beclin-1 expression and the number of autophagosome were significantly decreased, however, increasing Beclin-1 expression promoted the formation of autophagosomes and reduced the aggregation of Aβ (Singh et al., 2017). Microtubule-associated protein 1 light chain 3 (LC3), another specific gene to autophagy in mammalian, and

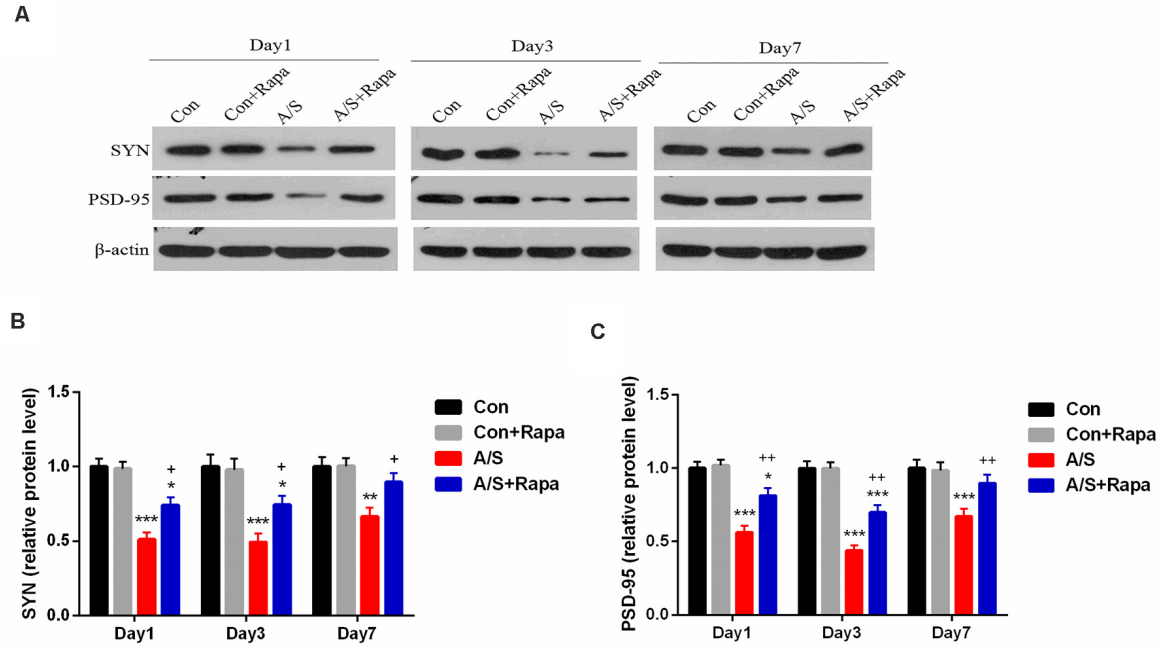

FIGURE 5 | Rapamycin treatment increased SYN and PSD-95 expression in the hippocampus. **(A)** Representative western blot illustrating the effects of rapamycin on the anesthesia/surgery-induced changes in hippocampal SYN and PSD-95 levels on days 1, 3 and 7 after anesthesia/surgery. **(B,C)** Rapamycin reversed anesthesia/surgery-induced decrease in SYN and PSD-95 levels in the hippocampus on days 1, 3 and 7 after anesthesia/surgery. Data are mean ± SEM (n = 6 per group). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. Con. $^{++}p < 0.01$, $^{+}p < 0.05$ vs. A/S. Con, control group; Con+Rapa, control group with rapamycin pretreatment; A/S, anesthesia/surgery group; A/S+Rapa, anesthesia/surgery group combined with rapamycin pretreatment.

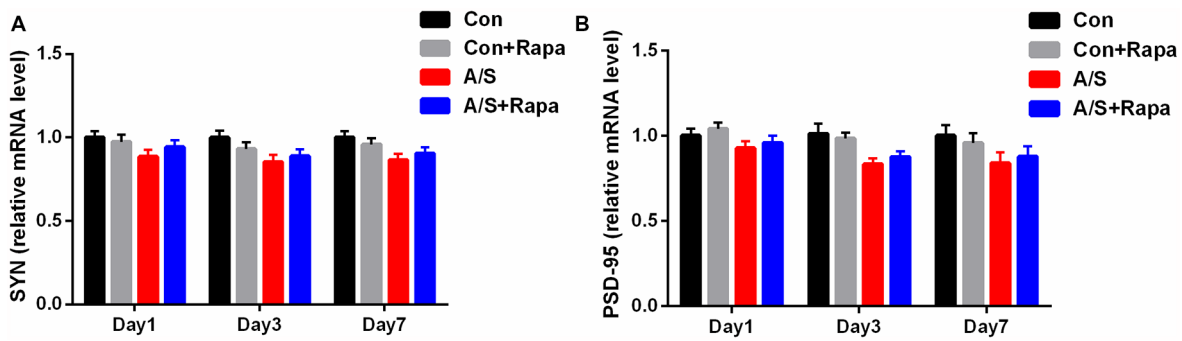

FIGURE 6 | Anesthesia/surgery and treatment with rapamycin were not significantly affected the mRNA levels of SYN and PSD-95. **(A,B)** SYN and PSD-95 mRNA levels in the hippocampus on days 1, 3, and 7 after anesthesia/surgery. Data are mean ± SEM (n = 6 per group). Con, control group; Con+Rapa, control group with rapamycin pretreatment; A/S, anesthesia/surgery group; A/S+Rapa, anesthesia/surgery group combined with rapamycin pretreatment.

expression of LC3-II is regarded as a marker of the activity of autophagy (Shpilka et al., 2011). Therefore, this study detected the expression of Beclin-1 and LC3-II, which were related to autophagy. Previous studies have suggested that the process of autophagy is negatively regulated by the activation of mTOR (Kim et al., 2011; Alers et al., 2012; Abada and Elazar, 2014). Numerous studies have shown that the role of mTOR signaling in protein homeostasis appears to be particularly important in learning and memory function (Bavley et al., 2018; Gao et al., 2018; Yuan et al., 2019). In the present study, we found that the levels of p-mTOR increased and Beclin-1 and LC3-II decreased in the hippocampus of mice undergone anesthesia/surgery. Furthermore, we showed that activation of autophagy following the administration of rapamycin reversed the cognitive deficits induced by anesthesia/surgery. Jiang et al. (2020) found that the levels of p-mTOR was increased following surgical trauma, an inhibitor of mTOR, rapamycin promoted autophagy activation. These data indicate that the mTOR-inhibited autophagy activation is involved in cognitive impairment induced by anesthesia/surgery.

Numerous studies have suggested that autophagy is involved in synaptic plasticity and neurotransmission

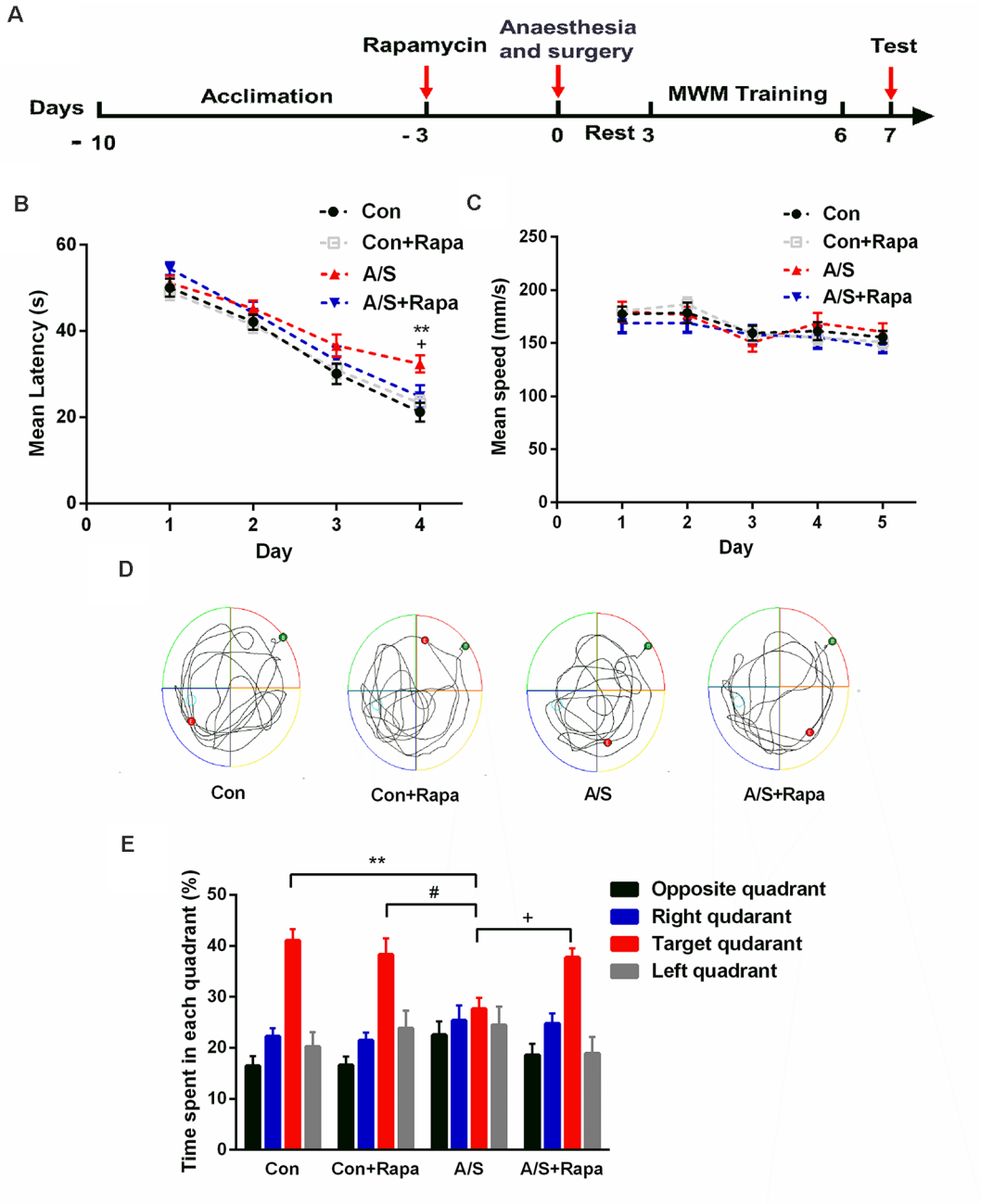

FIGURE 7 | Pretreatment with rapamycin alleviated the effects of anesthesia/surgery on reference memory in aged mice. **(A)** Schematic timeline of the experimental paradigm of surgery procedure and MWM test. After 3 days treatment with rapamycin, exploratory laparotomy was performed, training was conducted for 4 days followed by probe tests on day 7. **(B)** Escape latency to reach the hidden platform during the 4-day training; rapamycin reversed anesthesia/surgery-induced increase in escape latency. **(C)** Average swimming speed; there was no significant difference among the three groups. **(D)** Representative exploratory path of four groups of mice in the probe test. **(E)** The percentage of time spent in the target quadrant during the probe test; rapamycin reversed anesthesia/surgery-induced decrease in time spent in the target quadrant. Data are mean ± SEM (n = 12 per group). $^{**}p < 0.01$ vs. Con. $^{\#}p < 0.05$ vs. Con+Rapa. $^{+}p < 0.05$ vs. A/S. Con, control group; Con+Rapa, control group with rapamycin pretreatment; A/S, anesthesia/surgery group; A/S+Rapa, anesthesia/surgery group combined with rapamycin pretreatment.

(Hernandez et al., 2012; Nibuya et al., 2014; Takahashi et al., 2014). Autophagy-related synaptic dysfunction is associated with neurodegeneration, including Parkinson's disease and Alzheimer's (Harris and Rubinsztein, 2011; Nixon, 2013; Vijayan and Verstreken, 2017). Autophagy maintains homeostasis of synaptic proteins (Levine and Kroemer, 2019). Previous studies have shown that the age-related decline in memory is associated with a downregulation of ATG proteins (Shibata et al., 2006;

Cuervo, 2008; Rubinsztein et al., 2011). Thus, autophagy is vital to the homeostasis of synaptic proteins and memory consolidation (Shehata et al., 2012; Nixon, 2013). Glatigny et al. (2019) demonstrated that stimulation-induced autophagy was necessary to form new memories in the hippocampus and enhanced dendritic spine density and GluA1 and CAMKII a phosphorylation, markers of synaptic plasticity. Synaptic plasticity is considered important in learning and memory (Howland and Wang, 2008; Tian et al., 2015; Mansvelder et al., 2019). SYN and PSD-95 are involved in neuronal growth, process formation and synaptic plasticity regulation (Sifonios et al., 2009; Li et al., 2016) and play a key regulatory role in normal signal transmission among neurons in the central nervous system (Marco et al., 2017). Liu et al. (2017) demonstrated that cerebrolysin alleviated cognitive deficits induced by increasing the levels of synaptic plasticity-related proteins in the rat hippocampus, such as postsynaptic density protein 95 (PSD-95), protein kinase C subunit gamma (PKCγ). Tian et al. (2016) demonstrated that resveratrol limited diabetes-associated cognitive decline in rats by modulating hippocampal structural synaptic plasticity and enhancing SYN and GAP-43 expression in the hippocampus.

Our results showed that the levels of Beclin-1 and LC3-II, which are related to autophagy, and neuronal/synaptic plasticity-related proteins including SYN and PSD-95 decreased in the hippocampus of mice following anesthesia/surgery. At the same time, the results from the present study demonstrated the effect of rapamycin pretreatment on activation of autophagy, Beclin-1 and LC3-II expression was significantly up-regulated in the hippocampus, meanwhile, the levels of SYN and PSD-95 also increased. Thus, the inhibition of autophagy may probably be responsible for the hippocampal decline of SYN and PSD-95 following anesthesia and surgery.

Our study does have limitations. First, we did not identify specific mechanism of autophagy regulating SYN and PSD-95 in the anesthesia/surgery-induced cognitive decline. Further investigations are necessary to determine the specific molecular mechanisms between synaptic plasticity and autophagy. Second, rapamycin, as an immunosuppressive drug clinically, was observed and tested only for 7 days after anesthesia/surgery. The long-term effects of rapamycin on cognitive impairment after anesthesia/surgery need to be further explored.

In conclusion, our study demonstrated that anesthesia and surgery led tomTOR hyperactivity in the hippocampus, and disturbed neuronal/synaptic plasticity-related proteins homeostasis, possibly by inhibiting autophagy. These sequential pathological events may ultimately cause cognitive impairment. The results of the present study indicate that a novel signaling transduction mechanism is related to cognitive impairment after anesthesia/surgery. Rapamycin may be a potentially therapeutic agent for the treatment of anesthesia/surgery-induced cognitive impairment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Jiaxing University.

AUTHOR CONTRIBUTIONS

SG, SZ, HZ and JL designed the experiments. SG, SZ, XT, DP and WY performed the experiments. XT, YN, DP and SK contributed to data analyses. SG drafted this manuscript. HZ and JL reviewed and edited the manuscript. All authors interpreted the data, revised the manuscript, approved the final content, and read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Development and Validation of a Nomogram Based on Motoric Cognitive Risk Syndrome for Cognitive Impairment

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Objective: To develop and validate a prediction nomogram based on motoric cognitive risk syndrome for cognitive impairment in healthy older adults.

Methods: Using two longitudinal cohorts of participants (aged ≥ 60 years) with 4-year follow-up, we developed (n = 1,177) and validated (n = 2,076) a prediction nomogram. LASSO (least absolute shrinkage and selection operator) regression model and multivariable Cox regression analysis were used for variable selection and for developing the prediction model, respectively. The performance of the nomogram was assessed with respect to its calibration, discrimination, and clinical usefulness.

Results: The individualized prediction nomogram was assessed based on the following: motoric cognitive risk syndrome, education, gender, baseline cognition, and age. The model showed good discrimination [Harrell's concordance index (C-index) of 0.814; 95% confidence interval, 0.782–0.835] and good calibration. Comparable results were also seen in the validation cohort, which includes good discrimination (C-index, 0.772; 95% confidence interval, 0.776–0.818) and good calibration. Decision curve analysis demonstrated that the prediction nomogram was clinically useful.

Conclusion: This prediction nomogram provides a practical tool with all necessary predictors, which are accessible to practitioners. It can be used to estimate the risk of cognitive impairment in healthy older adults.

Keywords: motoric cognitive risk syndrome, slow gait, subjective cognitive decline, frailty, cognitive impairment, nomogram

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INTRODUCTION

The prevalence of age-related cognitive disorders, such as cognitive impairment and dementia for which few treatments are available, has been on the rise along with increasing world population, and these cognitive disorders have been shown to be independently associated with mortality in older people (Perna et al., 2015; Fogg et al., 2019). It is of paramount importance for clinicians to monitor cognitive change in older adults for timely taking steps to

delay or reverse these conditions (Brasure et al., 2018; Gates et al., 2019). Research on diagnosing these diseases at their earliest stages via risk factors may be the most successful strategy for individualizing cognition monitoring. Previous work had shown that physiological risk factors [i.e., slow gait (SG) speed (Chou et al., 2019), low grip strength (Chou et al., 2019; Liu et al., 2019), poor standing balance (Rolland et al., 2009), diabetes (Moran et al., 2019), and sustained hypertension (Walker et al., 2019)], behavioral risk factors (i.e., low physical activity; Brasure et al., 2018; Müller et al., 2018; Palta et al., 2019), demographic risk factors (i.e., age and gender; Nebel et al., 2018), environmental risk factors (i.e., air pollution; Paul et al., 2019), and genetic risk factors (Chang et al., 2018) are linked to cognitive impairment and dementia. However, only one domain of the above risk factors is poorly predictive of cognitive status, and their applicability and sensitivity in routine clinical practice are not convincing. For example, the concept termed "frailty" combines most physical performance tests and is considered as an early stage of disability (Fried et al., 2001). In comparison to participants free of frailty or cognitive impairment, the pooled hazard ratios (HRs) for dementia are the following: 3.83 for isolated cognitive impairment, 1.47 for isolated physical frailty, and 5.36 for their co-occurrence. The co-occurrence of cognitive impairment and physical frailty is a clinical marker of incident dementia (Grande et al., 2019). Furthermore, a syndrome called "motoric cognitive risk syndrome" (MCR), which is a combination of physiological risk factors (SG) and dementia risk assessments [subjective cognitive decline (SCD)], can be also used to identify older individuals at risk of cognitive impairment (Verghese et al., 2014). MCR is a novel approach that can be used even in resource poor settings and has incremental predictive validity for dementia as compared to its individual components and even after accounting for overlap with mild cognitive impairment (Verghese et al., 2014). However, no one method, including MCR, fulfills all requirements to be considered as a reference today.

Thus, the combined analysis of a prediction model based on a panel of risk factors, rather than individual analyses, may be the most promising approach to identify cognitive disorders. Nomograms, a pictorial representation of a complex mathematical formula (Grimes, 2008), use various variables to determine a prediction model that generates a probability of a clinical event, such as dementia or death. Several prediction nomogram models have been developed to predict dementia (Downer et al., 2016) or cognitive impairment (Zhou et al., 2020), but these models were mostly based on demographic risk factors, behavioral risk factors, and comorbidities. So far, prediction nomogram models are lacking that include information on markers that reflect the underlying disease process, especially in its early stages. Such markers include MCR or others. Although various kinds of risk factors have been considered and demonstrated, an optimal approach based on MCR or others as a predictive signature has yet to be developed. Therefore, this study aimed to develop and validate a prediction nomogram that incorporates MCR and other risk factors for individual preoperative prediction of cognitive impairment in healthy older adults.

MATERIALS AND METHODS

Development Cohort

Participants with baseline variables of interest from June 2011 to January 2016 were identified (n = 15,703) from the China Health and Retirement Longitudinal Study (CHARLS) (Zhao et al., 2014). Those who had missing values in one or more variables of interest (i.e., grip strength, gait speed, standing balance, chair stands, physical activity, SCD, weight loss, exhaustion) (n = 12,697), those 60 years or younger (n = 52), those who had stroke or memory-related diseases (n = 322) and disability (n = 1,040) were excluded from the analysis. In the remaining 1,592 participants, we further excluded those who were lost to follow-up (n = 154) and did not complete the cognitive test at baseline or follow-up (n = 96). We also classified participants as cognitive impairment if they were in the lowest 10% of the distribution of cognitive performance; thus, 165 participants with cognitive impairment at baseline were further excluded, as well. This resulted in a total 1,177 adults 60 years or older who received at least one follow-up. The process for selecting participants in the development cohort is shown in **Supplementary Figure 1**. Each participant signed a written informed consent form before taking part in the survey. Ethics approval for data collection in CHARLS was obtained from the Biomedical Ethics Review Committee of Peking University (IRB00001052-11015).

Validation Cohort

To ensure the adoption of best practices and international comparability or results, CHARLS is harmonized with leading international research studies in the Health and Retirement Study (HRS) (**Sonnega et al., 2014**) model. As a result, we used the cohorts in HRS for external validation. The validation (**Supplementary Figure 2**) cohort from the HRS consisted of 2,076 participants from April 2012 to April 2016 using the same criteria in the development cohort. This assessed whether the prediction nomogram could be used to predict cognitive impairment risk for healthy participants in the United States. HRS, which has been fielded every 2 years since 1992, is a nationally representative longitudinal survey that builds understanding of aging. To be consistent for validation, curation of HRS data followed the same process that was done for CHARLS.

Potential Predictors

The following 35 potential predictive variables were collected: demographic variables (8 variables), health status (16 variables), frailty and other physical performance test (8 variables), SCD, MCR, and baseline global cognition.

Demographic Variables

Demographic variables included age, gender, marital status, educational attainment, residence, body mass index (BMI), smoking status, and drinking habits. Gender was defined either as male or female. Marital status was defined as married if the participant was currently married and living with a spouse, or single if the participant was currently separated, divorced from a spouse, widowed, or never married. BMI measures (in kg/m^2)

were categorized using the following: <18.5 (thin), 18.5 to < 24 (normal), and ≥ 24 (overweight). Residence was defined either as urban or rural. Smoking and drinking habits were classified either as "never" or "current."

Health Status

Health status included hypertension, dyslipidemia, diabetes, cancer, chronic lung diseases, heart problems, arthritis, asthma, falls, hip fractures, near- and far-vision impairment, hearing problems, eating frequency, depressive symptoms, and instrumental activities of daily living (IADLs). Eating frequency was classified either as eating three times per day, eating more than three times per day, or eating less than three times per day. Depressive symptoms were assessed using the 10-item Center for Epidemiologic Studies Short Depression (CES-D) Scale, wherein a score greater than or equal to 10 indicates a significant depressive symptom (Andresen et al., 1994). Performance in IADLs was examined using five items: household chores, cooking, shopping, managing money, and taking medications. Participants were categorized as impaired if they scored more than 5 on the questionnaire. Responses for other items were dichotomized either as "yes" or "no."

Frailty and Other Physical Performance Test

Frailty was first described by Fried et al. (2001). It was measured using the physical frailty phenotype scale developed in the Cardiovascular Health Study and is objectively identified into five elements: weakness, SG, exhaustion, inactivity, and shrinking (Fried et al., 2001). Weakness was defined based on maximum grip strength of either hand, which is in the lowest 20th percentile of population distribution while adjusting for sex and BMI. SG was defined based on time to walk a 2.5-m course, which is in the lowest 20th percentile of population distribution while adjusting for sex and height. Exhaustion was identified if they answered "a moderate amount of time; 3–4 days" or "most of the time" to either of the two questions from the CES-D scale: "I could not get going" and "I felt everything I did was an effort." Inactivity was defined within the lowest level of physical activity as measured by the International Physical Activity Questionnaire. Shrinking was defined either as self-reporting a loss of five or more kilograms in the previous year or a BMI of 18.5 kg/m^2 or less. In summary, individuals with none were considered "non-frail"; meanwhile, those satisfying one or two criteria were considered "prefrail," and those with at least three to five criteria were defined as "frail." Lowest five chair stands were defined based on time to stand and sit five times as quickly as possible from the chair, which is in the lowest 20th percentile of population distribution while adjusting for sex and height. Lowest standing balance was defined if they were not able to maintain their feet in a side-by-side position for 10 s each.

MCR

MCR diagnosis was built with cognitive complaints but without significant functional impairment building on current operational definitions for mild cognitive impairment criteria (Petersen, 2011). As described above, it is a syndrome defined as SG combined with SCD. In our study, participants met the criteria for SCD if they answered "poor" to the following survey item: "How would you rate your memory at the present time? Would you say it is excellent, very good, good, fair, or poor?"

Follow-Up and Outcome

Under CHARLS, participants were observed once every 2 years and during follow-up visits using a complete cognitive test. Cognitive performance was calculated using three categories: mental intactness, episodic memory, and global cognition (Liu et al., 2019). Mental intactness included numerical ability (serial sevens), time orientation (date, month, year, day of the week, and season), and picture drawing (intersecting pentagon copying test), with scores ranging from 0 to 11. Episodic memory included immediate and delayed word recall, with scores ranging from 0 to 20. Global cognition was scored using the summation of the episodic memory and mental intactness scores, which ranges from 0 to 31. The main study outcome was cognitive impairment, which was used to categorize whether participants were in the lowest 10% of the distribution of global cognition during follow-ups. Furthermore, this population-based 10% cutoff point is a sensitive and specific maker of cognitive impairment (Ganguli et al., 1993) and has been used in other studies (Chandra et al., 1998; Yaffe et al., 2001; Mulsant et al., 2003).

Predictors Selection and Model Development

All 1,177 participants in the development cohort were included for predictor selection and model development. First, 35 variables were chosen into the selection process. least absolute shrinkage and selection operator (LASSO) method was applied to minimize the potential collinearity and overfitting of variables. Imputation for missing variables was considered if values were less than 20%. We used predictive mean matching and a dummy variable to impute numeric features and binary variables or factor features, respectively. The most useful predictors were selected using 1 standard error (1-SE) of the minimum criteria. The subsequent confidence interval (CI) identified by LASSO regression analysis was estimated using Kaplan–Meier method, and the log-rank test was used to compare survival curves. The Cox proportional hazard model was employed to develop a multivariate model and predict the 4-year cognitive impairment probability; meanwhile, a backward procedure based on Akaike information criterion (AIC) (Bozdogan, 1987) was used to construct the prediction model. The proportional hazards assumption was checked using graphical diagnostics based on scaled Schoenfeld residuals (Schoenfeld, 1982). To provide the clinician with a quantitative tool to predict individual probability of cognitive impairment, we built a multivariable Cox analysis–based nomogram in the development cohort.

Performance and Internal Validation of the Nomogram in the Development Cohort

Calibration curves were plotted to assess the nomogram. The Harrell's concordance index (C-index) was measured to

evaluate the discrimination performance of the nomogram. To calculate a relatively corrected calibration and C-index, bootstrapping validation (1,000 bootstrap resamples) was performed to the nomogram.

External Validation of the Nomogram

External validation was performed using the dataset from 2012 to 2016 in HRS. First, the total scores of each individual were calculated according to the constructed nomogram in the validation cohort. Second, we used the total scores as a factor to perform Cox regression analysis in this cohort, and finally, the C-index and calibration curve were derived based on the regression analysis.

Clinical Use

Decision curve analysis was performed to determine the clinical usefulness of the nomogram by quantifying the net benefits at different threshold probabilities in the validation dataset (Vickers et al., 2008).

Statistical Analysis

Results were presented as mean ± standard deviation or median (range) and number (percentage) for continuous variables and categorical variables, respectively. Continuous variables were explored for potential non-linear association with the risk of cognitive impairment using restricted cubic splines. LASSO Cox regression was done using the "glmnet" package. Multivariate Cox regression, nomograms, and calibration plots were done using the "rms" package. C-index calculation was performed using the "Hmisc" package. Internal validation was performed using the "rms" package. Decision curve analysis was performed with the "dca.R" function. The statistical significance levels for all analyses were two-sided, with a statistical significance of 0.05. Statistical analysis was performed with STATA version 15.0 (StataCorp, College Station, TX) and R software version 4.0.0[1].

RESULTS

Characteristics of Participants in the Development and Validation Cohorts

In the development cohort, 1,177 individuals (54.7% male) were included in the current study (**Supplementary Figure 1**). The median follow-up time was 48.0 months (range, 19.0–53.0 months) with a 4-year CI rate of 16.7%. The median age was 65.0 years (range, 60–90 years), and 17.6% of the participants reached higher education level. The median baseline cognition was 14.0, ranging from 6.0 to 28.0; 31.4% of the participants had SCD, through which 19.9% and 6.7% had SG and MCR, respectively. The characteristics of participants in the development cohorts are listed in **Table 1**. Similarly, the procedure and exclusion criteria are depicted in **Supplementary Figure 1**.

[1]http://www.r-project.org

TABLE 1 | Characteristics of participants in the development and validation cohorts.

	Development cohort (*n* = 1,177)	Validation cohort (*n* = 2,076)
Cognitive impairment		
Yes	196 (16.7)	388 (18.7)
No	981 (83.3)	1,688 (81.3)
Follow-up time (months), median (range)	48.0 (19.0–53.0)	48.0 (14.0–63.0)
Age (years), median (range)	65.0 (60.0–90.0)	73.0 (65.0–97.0)
Gender, no. (%)		
Male	644 (54.7)	839 (40.4)
Female	533 (45.3)	1,237 (59.6)
Education, no. (%)		
High school or less (≤12 years)	970 (82.4)	1,009 (48.6)
College or higher (>12 years)	207 (17.6)	1,067 (51.4)
Baseline cognition, median (range)	14.0 (6.0–28.0)	24.0 (18.0–34.0)
Subjective cognitive decline, no. (%)		
Yes	369 (31.4)	55 (2.6)
No	808 (68.6)	2,021 (97.4)
Slow gait, no. (%)		
Yes	234 (19.9)	344 (16.6)
No	943 (80.1)	1,732 (83.4)
Motoric cognitive risk syndrome		
Healthy	653 (55.5)	1,693 (81.6)
Only subjective cognitive decline	290 (24.6)	39 (1.9)
Only slow gait	155 (13.2)	328 (15.8)
Motoric cognitive risk syndrome	79 (6.7)	16 (0.7)

Predictors Selection and Development of an Individualized Prediction Model

Thirty-five variables were included in the LASSO regression. After LASSO regression selection (**Figures 1A,B**), five variables remained significant predictors of cognitive impairment, which included education, gender, baseline cognition, age, and MCR. Furthermore, we found that there was sufficient evidence for a linear relationship between age, baseline cognition, and risk of cognitive impairment. Furthermore, inclusion of these five variables in a Cox regression model resulted to independently statistically significant predictors of cognitive impairment for all variables. Hence, these were included in final prediction model after a backward procedure based on AIC. These variables included the following: MCR (HR, 1.952; 95% CI, 1.205–3.160; $P = 0.007$), education (HR, 0.907; 95% CI, 0.879–0.936; $P < 0.001$), gender (female vs. male) (HR, 1.568; 95% CI, 1.166–2.110; $P = 0.003$), age (HR, 1.042; 95% CI, 1.019–1.065; $P < 0.001$), and baseline cognition (HR, 0.792; 95% CI, 0.758–0.828; $P < 0.001$) (**Table 2**). The nomogram that integrated all significant independent factors for cognitive impairment in the development cohort is shown in **Figure 2**. Similarly, the results of the univariate analysis are listed in **Supplementary Table 1**.

Performance and Internal validation of Nomogram in the Development Cohort

The nomogram was internally validated using the bootstrap-corrected calibration plot and Harrell C statistic, which resulted to a Harrell C-index for cognitive impairment prediction of

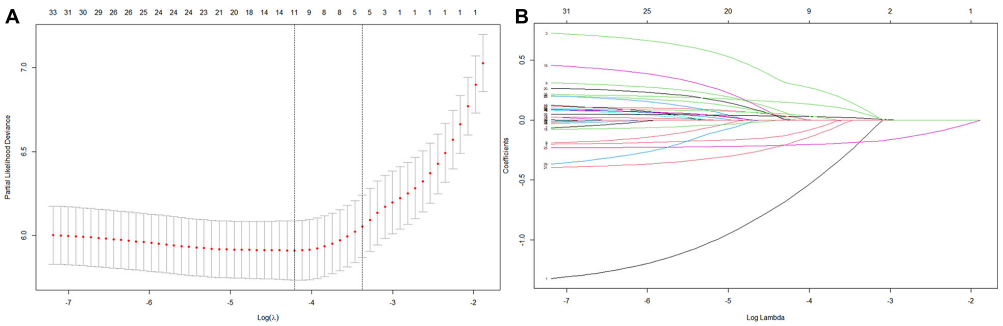

FIGURE 1 | Variables selection using the least absolute shrinkage and selection operator (LASSO) Cox regression model. **(A)** Tuning parameter (λ) selection in the LASSO model used 10-fold cross-validation via 1 standard error (SE) of the minimum criteria (the 1-SE criteria). **(B)** LASSO coefficient profiles of the 35 variables.

0.814 (95% CI, 0.782–0.835). The bootstrap-corrected calibration plot for the probability of 4-year survival showed an optimal agreement between the prediction by nomogram and actual observation (**Figure 3A**).

Comparison of Predictive Accuracy Between the Nomogram and MCR

A reference model based on MCR alone yielded a C-statistic of 0.615 (95% CI, 0.571–0.645), which is significantly worse as compared with prediction nomogram ($P < 0.001$).

External Validation of Predictive Accuracy of the Nomogram for Cognitive Impairment

For the validation cohort, we studied 2,076 individuals (**Supplementary Figure 2**). The median follow-up time was 48.0 months (range, 14.0–63.0 months), with 18.7% of participants suffering from cognitive impairment during follow-up. The baseline characteristics of the CHARLS and HRS participants were comparable, whereas the HRS participants were older and more educated and reported less SCD, slower gait, and MCR on average (**Table 1**). The Harrell C-index of the nomogram for predicting cognitive impairment was 0.772 (95% CI, 0.776–0.818), and the calibration curve showed good agreement between prediction and observation in the 4-year cognitive impairment probability (**Figure 3B**).

TABLE 2 | Multivariable Cox regression model for predicting development of cognitive impairment in 1,177 participants.

Independent variable	Cognitive impairment HR (95% CI)	*P*-value
MCR		
Healthy	**Ref.**	
Subjective cognitive decline	1.564 (1.121–2.183)	0.009
Slow gait	1.842 (1.205–2.817)	0.005
Motoric cognitive risk syndrome	1.952 (1.205–3.160)	0.007
Age	1.042 (1.019–1.065)	<0.001
Education	0.907 (0.879–0.936)	<0.001
High school or less (≤12 years)	**Ref.**	
College or higher (>12 years)	0.232 (0.094–0.570)	0.001
Baseline cognition	0.792 (0.758–0.828)	<0.001
Gender (female vs. male)	1.568 (1.166–2.110)	0.003

CI, confidence interval; HR: hazard ratio; Ref., reference.

Clinical Use

The decision curve analysis for the nomogram is presented in **Supplementary Figure 3**. The net benefit curves for cognitive impairment over 4 years show that there is higher net benefit than strategies based on considering either no participants or all participants for intervention at risk thresholds up to approximately 80%.

DISCUSSION

We developed and validated a prediction nomogram for cognitive impairment in cognitively healthy older adults. The nomogram incorporates five items: MCR, age, education, baseline cognition, and gender. The nomogram can be used to calculate the 4-year risk of cognitive impairment and successfully stratified participants according to their individual risks. All predictors included in the nomogram were easy to assess and readily available. There were also no additional expensive tests needed, such as brain imaging. Incorporating the MCR and other risk factors into an easy-to-use nomogram facilitated the preoperative individualized prediction of cognitive impairment.

The combined analysis of a prediction nomogram model based on MCR and other important risk factors, rather than individual components, resulted in better predictive performance to identify cognitive impairment. MCR syndrome provides incremental validity over its individual components (Verghese et al., 2014). However, age, educational attainment, baseline cognition, and gender are also strongly associated with risk of cognitive impairment. Although MCR has previously been described by researchers for a screening marker of cognitive impairment, our study has some important differences from this previous work. In this analysis, we estimated 4-year risk of

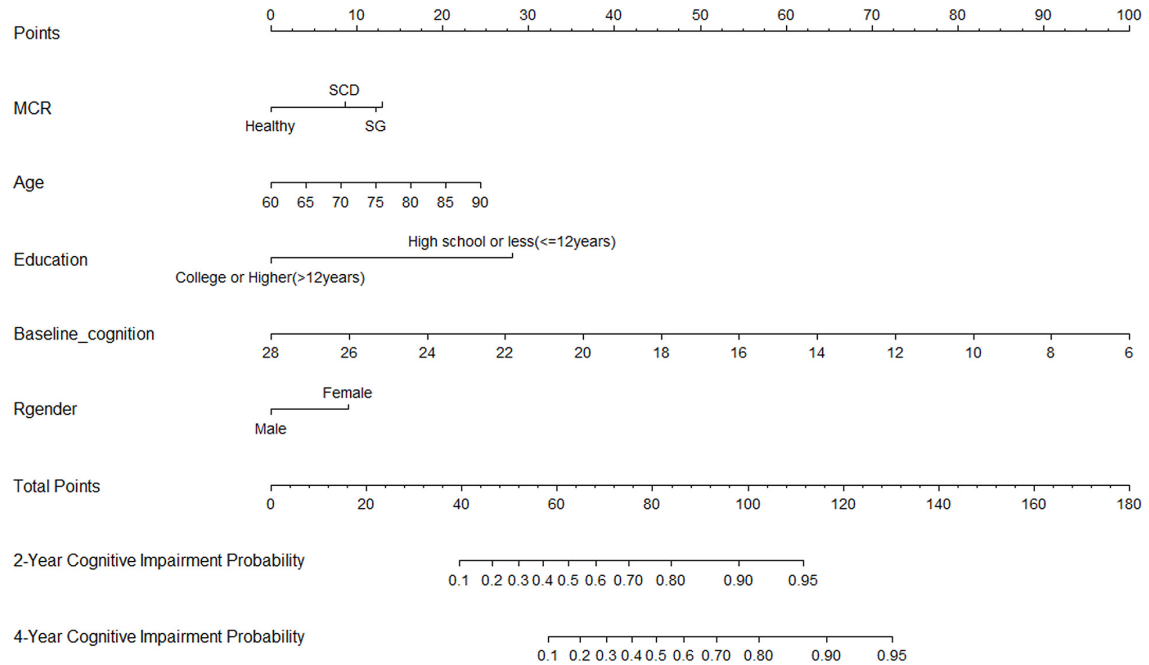

FIGURE 2 | Nomogram for predicting the 4-year cognitive impairment probability. To calculate the cognitive impairment probability for a specific patient, locate patient's MCR status, and draw a line straight upward to the Points axis to determine the score associated with that status. Repeat the process for age, education status, baseline cognition, and gender; sum the scores for each factor; and locate this sum on the Total Points axis. Then, draw a line straight down to the corresponding 2- or 4-year cognitive impairment probability axis to find the predicted cognitive impairment probability.

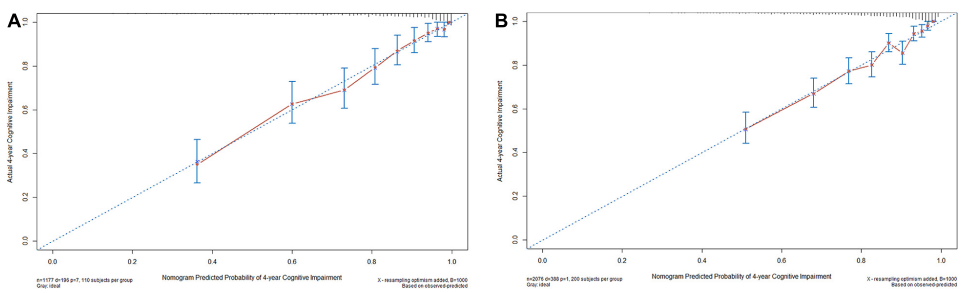

FIGURE 3 | The internal **(A)** and external **(B)** calibration curves of the nomogram. Nomogram-predicted probability and observed frequency over 4 years for cognitive impairment among participants with normal cognition at baseline were plotted in the *x*- and *y*-axis, respectively. The gray line indicates the ideal plot for the calibration curve, where the nomogram-predicted probabilities perfectly match the observed probabilities in all subgroups.

cognitive impairment and incorporated these into a simple point-scoring scheme for predicting cognitive impairment risk that has significant practical utility.

Previous studies have investigated MCR (Sonnega et al., 2014; Verghese et al., 2019) or frailty (Panza et al., 2019; Wallace et al., 2019) as biomarkers of cognitive impairment and dementia in cognitively healthy older adults. However, this study noted that frailty showed enough predictive strength on the basis of univariable association with cognitive impairment but not included in the prediction nomogram; however, the rejection of important predictors may be a result of collinearity or confounding by other predictors (Collins et al., 2015). As a qualitative prediction model, MCR can be easily measured. Our study further demonstrated that MCR was associated with cognitive impairment, and the MCR-based nomogram was identified as a useful tool in the selection of high-risk patients for early intervention studies and applications of personalized medicine.

For the prediction nomogram, 35 variables were reduced to five predictors by shrinking the regression coefficients with the LASSO method. This method not only surpasses the method of choosing predictors on the basis of the strength of their univariable association with outcome (Boyd, 2005), but also enables the panel of selected features to be combined into a prediction nomogram. Multimarker analyses that incorporate individual markers into marker panels have been incorporated in recent studies (Exalto et al., 2013;

Downer et al., 2016; Li et al., 2018; Licher et al., 2019; Zhou et al., 2020). Similarly, the prediction nomogram that combined multiple individual risk factors demonstrated adequate discrimination in the development cohort (C-index, 0.814; 95% CI, 0.782–0.835); meanwhile, maintenance was observed in the validation cohort (C-index, 0.772; 95% CI, 0.776–0.818). The opportunity to undertake an external validation in HRS, which was conducted in a different geographical location, corroborated our findings. More importantly, different predictors were distributed in the development and validation cohort, which it only slightly affected the performance of nomogram in the validation cohort, emphasizing that the nomogram is robust.

Given that incidence of cognitive impairment was comparable in the two cohorts, the nearly equal discrimination demonstrated that the prediction nomogram was stable for prediction and could be applied directly in the validation cohort. This involved omitting the process of adjusting intercept and regression coefficients regarding the nomogram construction, as well. In a recent study that investigated the dementia risk of using age, history of stroke, SCD, and need for assistance with finances or medication, the derived accuracy of combined risk factor was 78%. This is lower than the C-index of the prediction nomogram we constructed. The most important and final dispute for the use of the nomogram was based on the need to interpret individual need of additional intervention. However, the performance of nomogram based on MCR, discrimination, and calibration could not capture the clinical consequences of a particular level of discrimination or degree of miscalibration (Localio and Goodman, 2012; Collins et al., 2015; Van Calster and Vickers, 2015). Therefore, we assessed whether decisions based on the nomogram would improve individual outcomes to justify the clinical usefulness. As a result, decision curve analysis was applied in this study, and it offers insights into outcomes based on threshold probability, from which the net benefit could be derived. The decision curve showed that if the threshold probability of a patient or doctor is 0–80%, using the prediction nomogram in the current study to predict cognitive impairment adds more benefit than either the treat-all-patients schedule or the treat-none schedule.

Study limitations include the non-consideration of environmental risk factors or genetic risk factors on the assessment of 35 candidate predictors. However, it is yet to be decided whether simply building a model that applies the easy-to-get features to predict outcomes directly is preferable to combined genetic factors. Second, we used a regularization method (LASSO) that automatically selects and subsequently shrinks effect sizes of important predictors. This may have led to some underestimation of predictor effects in the development data.

CONCLUSION

In conclusion, this study presented a nomogram that incorporated both MCR and other risk factors and can be conveniently used to facilitate the preoperative individualized prediction of cognitive impairment in cognitively healthy older adults.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethics approval for data collection in Charls was obtained from the Biomedical Ethics Review Committee of Peking University (IRB00001052-11015). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and CL designed this study and drafted the manuscript. YL and KW acquired the data. YL, LJ, and NG performed the statistical analysis, assisted by LF and CL. KW and LF reviewed the manuscript. All authors approved the final version for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.618833/full#supplementary-material

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REVIEW
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Delirium: A Marker of Vulnerability in Older People

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Delirium is an acute neuropsychiatric syndrome and one of the most common presenting symptoms of acute medical illnesses in older people. Delirium can be triggered by a single cause, but in most cases, it is multifactorial as it depends on the interaction between predisposing and precipitating factors. Delirium is highly prevalent in older patients across various settings of care and correlates with an increased risk of adverse clinical outcomes. Several pathophysiological mechanisms may contribute to its onset, including neurotransmitter imbalance, neuroinflammation, altered brain metabolism, and impaired neuronal network connectivity. Several screening and diagnostic tools for delirium exist, but they are unfortunately underutilized. Additionally, the diagnosis of delirium superimposed on dementia poses a formidable challenge – especially if dementia is severe. Non-pharmacological approaches for the prevention and multidomain interventions for the treatment of delirium are recommended, given that there is currently no robust evidence of drugs that can prevent or resolve delirium. This article aims to review the current understanding about delirium in older people. To achieve this goal, we will describe the epidemiology and outcomes of the syndrome, the pathophysiological mechanisms that are supposed to be involved, the most commonly used tools for screening and diagnosis, and prevention strategies and treatments recommended. This review is intended as a brief guide for clinicians in hospital wards to improve their knowledge and practice. At the end of the article, we propose an approach to improve the quality of care provided to older patients throughout a systematic detection of delirium.

Keywords: delirium, elderly, frailty, Atypical symptoms, confusion

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INTRODUCTION

Several years ago, Lipowski wrote: "Acute medical confusion as a presenting symptom holds a central position in the medicine of old age [. . .] acute confusion is a far more common herald of the onset of physical illness in an older person, than are, for example, fever, pain or tachycardia. The elderly, especially the very old, are uniquely prone to delirium as a consequence of almost any physical illness or of intoxication with even therapeutic doses of commonly used drugs [. . .] Failure to diagnose delirium and to identify and treat its underlying causes may have lethal consequences for the patient, since it may constitute the most prominent presenting feature of myocardial infarction, pneumonia, or some other life-threatening physical illness" (Lipowski, 1983, 1989).

Reading these sentences, the reader sees clearly how delirium has long been recognized as one of the most important components of the geriatric symptomatology, and a prognostic marker of older patients with acute illnesses. Nevertheless, in everyday practice, physicians and nurses often lack both knowledge and training about this syndrome (Bellelli et al., 2014b), referring to it with heterogeneous jargon (Teodorczuk et al., 2013). Moreover, because countless junior doctors still underestimate the impact of this condition in older people, they lack perspective about the implementation of preventative and therapeutic measures across settings of care. Finally, the lack of definite biomarkers and pharmaceutical agents specific for delirium contribute to the low impact of delirium on medical culture.

The scope of this article is to review the current understanding about delirium in older people, describing its epidemiology and outcomes, the pathophysiological mechanisms that are supposed to be involved, the most commonly used tools for screening and diagnosis, and prevention and treatments recommended. A particular focus is placed on the diagnostic challenge of delirium superimposed on dementia (DSD). We hope that this review will serve as a brief guide for clinicians working in acute hospitals to improve the quality of care provided toward their older patients.

DEFINITION AND CAUSES OF DELIRIUM

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), (American Psychiatric Association, 2013) defines delirium as a neuropsychiatric syndrome that encompasses different signs and symptoms, especially disturbances in attention and awareness. This set of signs and symptoms represents the altered reaction of the CNS and its functioning when dealing with acute medical conditions, intoxication or withdrawal of medications, surgery, and electrolyte or metabolic imbalances (American Psychiatric Association, 2013). Delirium can be triggered by a single cause, but in most cases, it is multifactorial, resulting from the interaction between predisposing and precipitating factors. The higher the burden of the predisposing factors, the lower the magnitude of the precipitating factors required to cause delirium (Inouye and Charpentier, 1996; Inouye et al., 2014).

According to a landmark study by Inouye and Charpentier (1996), delirium has four main predisposing and five major precipitating factors. The former includes dementia/cognitive impairment, sensory deprivation, dehydration, and severity of an acute occurring illness, while the latter encompass poor nutritional status/malnutrition, use of physical restraints, the recent prescription of three or more new medications, urinary bladder catheterization, and other iatrogenic factors (Inouye and Charpentier, 1996). However, a number of other predisposing factors have been identified since that study. A systematic review and meta-analysis including 11 articles (total study population = 2,338 patients), identified dementia, illness severity, visual impairment, urinary catheterization, low albumin levels, and increased length of hospital stay as risk factors for delirium (Ahmed et al., 2014). Cognitive impairment and dementia were also prominent risk factors for delirium in a systematic review on patients with hip fracture and in another systematic review on patients from Intensive Care Unit (ICU) (Oh et al., 2015; Zaal et al., 2015). Recently, a study by Bowman et al. (2020) identified 55 risk factors that were predictive of delirium occurring in the community or recorded in emergency hospitalisation. These included cognitive impairment or mental illness, psychoactive drugs, frailty, infection, hyponatraemia and anticholinergic drugs (Bowman et al., 2020). Recent studies are suggesting the SARS-CoV-2 infection may be a potential cause of delirium in older people admitted to hospital wards (Zaal et al., 2015).

EPIDEMIOLOGY

Delirium is common in older people across various settings of care. A recent systematic review and meta-analysis identified 33 studies that evaluated the occurrence of delirium in medical inpatients over time (Gibb et al., 2020). Only studies using internationally accepted diagnostic criteria for the diagnosis were included. Overall, delirium prevalence was 23% (95% CI 19–26%), with variations related to diagnostic criteria used (highest in DSM-IV, lowest in DSM-5), a proportion which was similar to a systematic review performed 14 years earlier (Siddiqi et al., 2006). There are no systematic reviews about its incidence in surgical patients, but studies demonstrate that delirium is a common surgical complication among older adults, with incidence of 15–25% after major elective surgery and 50% after high-risk procedures such as hip-fracture repair and cardiac surgery (Marcantonio, 2017). Delirium occurrence might be even higher in the ICU. A recent systematic review and meta-analysis including 48 studies from medical, surgical, or specialty ICUs (total number of patients = 27,342) found an overall pooled prevalence of 31% (Krewulak et al., 2018). However, in mechanically ventilated patients, delirium occurrence can range from 60 to 80% (Ely et al., 2001a).

The burden of this syndrome is also relevant in the post-acute care setting and rehabilitation facilities, where delirium prevalence is about 14–18% (Morandi et al., 2014; Bellelli et al., 2016), and in nursing homes, where delirium prevalence, according to a review, can range from 1.4 to 70% (de Lange et al., 2013). More recently, a study with 1,454 patients from 71 nursing homes, found a delirium point-prevalence of 36.8% (Morichi et al., 2018). Delirium is thought to be less frequent in the community (1–3%) (Inouye et al., 2014), but studies are limited by the methods to detect it and the selection criteria. Moreover, it should be considered that delirium onset usually leads the patient to be referred to an emergency room, a setting in which this syndrome is present in 8 to 17% of older patients, and up to 40% in nursing home residents (Inouye et al., 2014).

OUTCOMES

Patients with delirium show an increased risk of developing poor clinical outcomes, including increased likelihood of nursing home placement and death (Witlox et al., 2010;

Inouye et al., 2014). When experienced during a hospitalization, delirium increases 2-year mortality risk by approximately two-fold after adjusting for age, gender, chronic diseases, and dementia (Witlox et al., 2010). The negative effect of delirium on mortality has also been shown in patients with SARS-CoV-2 infection (Marengoni et al., 2020; Rebora et al., 2020). Importantly, the longer its duration, the higher the risk of death. A study performed in a group of older patients who underwent surgery after hip fracture showed that post-operative delirium incremented the hazard ratio of 6-month death by 17% per day of experiencing the syndrome, after adjusting the model for potential confounders (Bellelli et al., 2014a). Delirium may also increase the likelihood of developing cognitive impairment and/or progress to dementia. Recently, a systematic review by Goldberg et al. performed a meta-analysis of 24 studies, with 3,562 subjects experiencing delirium and 6,987 controls who did not. The authors found that delirium and the development of long-term cognitive deterioration were significantly associated (Goldberg et al., 2020). All studies demonstrated that patients who developed delirium also displayed worse cognitive performances at follow-up (Goldberg et al., 2020).

The emotional distress of patients, caregivers and healthcare workers is a further negative outcome of delirium, since it may affect not only patients, but also their family members and the members of the hospital and nursing home staff (Mc Donnell and Timmins, 2012; Morandi et al., 2015; Schmitt et al., 2019). Interestingly, a study described that these three categories (i.e., patients, family caregivers, and nurses) share three common themes of delirium-related burden, namely symptom burden, emotional, and situational burden (Schmitt et al., 2019). These findings support the theory of delirium as a shared experience, indirectly suggesting that multidisciplinary system-wide strategies may better address delirium-associated interpersonal consequences (Schmitt et al., 2019).

PATHOPHYSIOLOGY

The pathophysiology of delirium still remains speculative and it may represent a diverse range of pathobiology processes rather than a single entity. From an historic perspective, delirium has been viewed as a disorder of several neurotransmitters, including acetylcholine, melatonin, dopamine, norepinephrine, glutamate, 5-hydroxytryptamine or serotonin, histamine, and/or gamma-aminobutyric acid (Maldonado, 2018). However, the pathophysiology of delirium is more complex than that. Here, we present only some of the most promising current theories on this topic. One theory proposes that delirium is the result of the combination of disorders in the neurotransmission, a failure in integrating and processing sensory signals and motor effectors, and a breakdown in cerebral network connectivity (Maldonado, 2018). It postulates that several patient-specific factors interact to develop delirium, including neuroinflammation, excess of "oxidative stress," "neuroendocrine dysfunction" and "circadian rhythm or melatonin dysregulation" (Maldonado, 2018). For example, a peripheral infection or surgery may activate inflammatory cytokines and other mediators in the blood that can cross the blood brain barrier or reach the brain parenchyma through other routes (e.g., the vague nerve), and here activate the microglia cells and astrocytes (Wilson et al., 2020). The alterations of the body composition (i.e., a reduction of the lean mass and an increase of the fat mass) that commonly occur in older individuals during their life, might also play a role as additional source of inflammatory stimulus (through endocrine secretion of pro-inflammatory adipokines), amplifying the magnitude of the response (Bellelli et al., 2017).

This sequence of events leads to a misalignment in neuronal function, synaptic impairment, and the subsequent onset of the multi-faceted symptomatology of delirium, which involves neurological deficits, and alterations in the behavior and cognition. Importantly, neuroendocrine dysfunctions, excess of oxidative stressors and defect of melatonin can also contribute to neuroinflammation of the brain (Cunningham et al., 2009; Cerejeira et al., 2012), thus indirectly maintaining the synaptic dysfunction.

More recently, other mechanisms underpinning delirium onset have been proposed. The brain bioenergetics insufficiency is one of them. Neurons and astrocytes both require massive amount of glucose supplied by the microvasculature to generate adenosine triphosphate (ATP) by glycolysis (Wilson et al., 2020). However, several acute illnesses may impair the supply of glucose to the brain. For instance, lung infections can cause hypoxemia, reducing neuronal energy metabolism through an impaired mitochondrial production of ATP (Wilson et al., 2020), and hemodynamic shock can impair blood flow to the brain and thus impair glucose supply. Moreover, even microcapillary and neurovascular dysfunctions, as well as systemic hypoglycemia can all provoke insufficient glucose supply and, thus, delirium and coma (Wilson et al., 2020). Evidence to support this hypothesis comes from studies on hip fracture and medical patients with delirium (Caplan et al., 2010; Kealy et al., 2020). However, studies with Fluorodeoxyglucose – Positron Emission Tomography (FDG-PET) imaging in mice and humans have shown that glucose uptake is substantially reduced with sepsis and overall glucose metabolism is impaired during delirium (Semmler et al., 2008; Hölscher, 2019).

According to the first theory, delirium is the final product of a breakdown in the efficiency of brain network that cannot compensate for injury and diseases. For instance, in older individuals with high-grade neurodegeneration, developing insults and stressors may lead to a derangement of function between brain areas and thus to delirium (Shafi et al., 2017). This mechanism is supported by studies showing that delirious patients display atrophy in the amygdala and decreased gray matter volumes in some brain areas (Rolandi et al., 2018), that delirium duration is increased in individuals with loss of integrity in the inter-hemispheric corpus callosum (Morandi et al., 2012b), and that abnormalities in the hippocampus, thalamus, basal forebrain and cerebellum are associated with incidence and severity of delirium (Cavallari et al., 2016).

Figure 1 summarizes the interaction between predisposing and precipitating factors leading to delirium and to some adverse clinical outcomes.

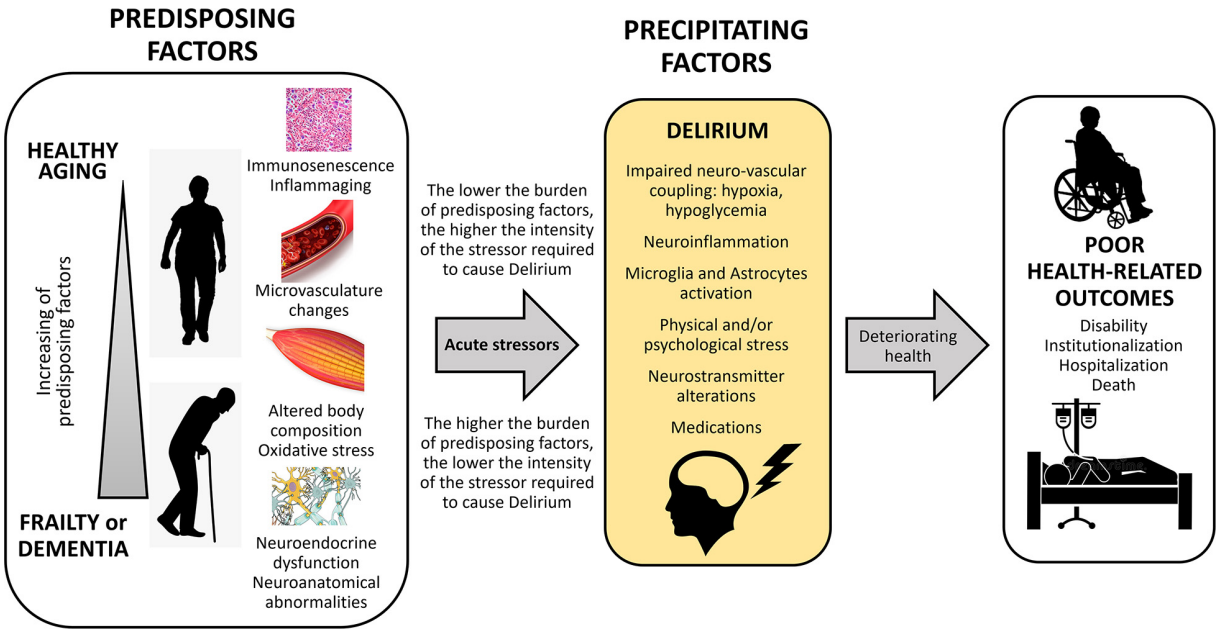

FIGURE 1 | Conceptual framework of predisposing and precipitating factors leading to delirium after an acute stressor, and hesitating in poor health-related outcomes.

DIAGNOSIS OF DELIRIUM

Clinical Features of Delirium

Delirium is recognized by a constellation of symptoms. Inattention, disorganized thought, altered consciousness and other multiple cognitive domains represent the key features (American Psychiatric Association, 2013). However, hallucinations, delusions, incoherent speech, inappropriate behavior, emotional lability, and alterations of the sleep–wake cycle can also be present (Inouye et al., 2014).

There are at least three psychomotor subtypes of delirium, i.e., hypoactive, hyperactive, and mixed. The former is characterized by a withdrawal from interaction with the outside world, sluggishness (or lethargy) and reduced psychomotor activity; the hyperactive subtype presents restlessness, agitated behavior and/or aggressiveness; and the mixed subtype is characterized by the transition from hyperactivity to hypoactivity or vice versa. Meagher et al. (2014) described a non-hyperactive-non-hypoactive subtype of delirium, which is characterized by normal level of psychomotor activity and no fluctuations between hyperactive and hypoactive subtypes. It is still unclear which of these motor subtypes may underlie different causes of delirium. However, various studies suggest that the hypoactive and mixed subtypes are more likely to develop in frail older individuals, thus correlating with a more severe prognosis (Bellelli et al., 2007, 2018). More importantly, different psychomotor subtypes may have distinct risk factors, indirectly suggesting that they should be different clinical phenomena (Morandi et al., 2017). A potential role for altered noradrenergic activity in influencing the arousal level and therefore the delirium psychomotor subtype (Hahn et al., 1995; Matthews et al., 2002) is a longstanding object of speculation. However, further studies are needed to clarify this issue.

Diagnostic Criteria and Screening Tools

Delirium is essentially a clinical diagnosis. Currently, the DSM-5th edition and the International Statistical Classification of Diseases and Related Health Problems, 10th revision criteria represent the gold standard for the diagnosis (**Table 1**). However, a number of screening tools for delirium have been developed in recent years to help physicians in its detection. Here, we will report two of the most commonly used.

The Confusion Assessment Methods (CAM) has been developed by Inouye et al. (1990). It includes an algorithm based on 4 core features of delirium: (1) acute change or fluctuating course, (2) inattention, and either or both (3) disorganized thinking, and (4) alteration of consciousness. The CAM has been validated in a number of high-quality studies showing high sensitivity (94–100%), and specificity (89–95%). There is also a CAM-ICU version for use with non-verbal mechanically ventilated patients (Ely et al., 2001b). However, preliminary training is critical for its use. In fact, one study has shown that without preliminary training of the examiners, the rate of underdiagnoses was unacceptably high (Inouye et al., 2001). Sensory deficits, dementia, the hypoactive psychomotor subtype of delirium and old age were recognized as risk factors for the under-recognition (Inouye et al., 2001).

The 4AT test is a relatively new tool proposed by Maclullich and colleagues (available at[1]). Importantly, the test does not

[1]www.the4at.com

require special training and generally takes less than 2 min to be completed; furthermore, it can be performed also in subjects with visual or hearing impairment and in "untestable" individuals with severe agitation or drowsiness (Bellelli et al., 2015a). The 4AT encompasses four items: alertness (item 1), the Abbreviated Mental Test – 4 (AMT4, item 2), attention (tested with months of the year backwards, MOTYB, item 3), acute change in mental status or its fluctuation (item 4). Recently, a prospective randomized multicenter study demonstrated that the 4AT displays a higher sensitivity compared to the CAM (76% [95% CI 61–87%] vs. 40% [95% CI 26–57%], respectively) (Shenkin et al., 2019). Conversely, the CAM had a higher specificity (100% [95% CI 98–100%] for CAM vs. 94% [95% CI 92–97%] for 4AT, respectively) (Shenkin et al., 2019). The results of this study suggest that 4AT may be proposed as a tool to improve detection of delirium as well as CAM, or that either tool may have a role in screening depending on the setting and purpose.

Barriers to Delirium Recognition

As already mentioned, delirium has been recognized for at least two millennia as a dangerous condition, but still remains underdiagnosed. There are many possible explanations for this. One is that medical culture does not regard delirium as an important topic. For instance, many Academic courses for undergraduates, medical doctors, and nurses do not include delirium in their programs. Additionally, the medical textbooks are usually arranged by disorders while disregard syndromes like delirium. Another reason is that delirium has received various clinical labels such as "acute confusion," "acute organic brain syndrome," "brain failure," "intensive care psychosis," "toxic encephalopathy." The use of these terms actually impede the

TABLE 1 | The criteria used to diagnose delirium: the Diagnostic and Statistical Manual of Mental Disorders (DSM) -5th edition and the International Statistical Classification of Diseases and Related Health Problems (ICD), 10th revision criteria.

	DSM 5th		ICD 10th
A	A disturbance in attention (i.e., reduced ability to direct, focus, sustain, and shift attention) and awareness (reduced orientation to the environment).	A	Clouding of consciousness, i.e., reduced clarity of awareness of the environment, with reduced ability to focus, sustain, or shift attention.
B	The disturbance develops over a short period of time (usually hours to a few days), represents a change from baseline attention and awareness, and tends to fluctuate in severity during the course of a day.	B	Disturbance of cognition, manifest by both: impairment of immediate recall and recent memory, with relatively intact remote memory; disorientation in time, place or person.
C	An additional disturbance in cognition (e.g., memory deficit, disorientation, language, visuospatial ability, or perception).	C	At least one of the following psychomotor disturbances: rapid, unpredictable shifts from hypoactivity to hyperactivity; increased reaction time; increased or decreased flow of speech; enhanced startle reaction.
D	The disturbances in Criteria A and C are not better explained by another pre-existing, established, or evolving neurocognitive disorder and do not occur in the context of a severely reduced level of arousal, such as coma.	D	Disturbance of sleep or the sleep-wake cycle, manifest by at least one of the following: insomnia, which in severe cases may involve total sleep loss, with or without daytime drowsiness, or reversal of the sleep-wake cycle; nocturnal worsening of symptoms; disturbing dreams and nightmares which may continue as hallucinations or illusions after awakening.
E	There is evidence from the history, physical examination, or laboratory findings that the disturbance is a direct physiological consequence of another medical condition, substance intoxication or withdrawal (i.e., due to a drug of abuse or to a medication), or exposure to a toxin, or is due to multiple etiologies. Specify whether Substance intoxication delirium: This diagnosis should be made instead of substance intoxication when the symptoms in Criteria A and C predominate in the clinical picture and when they are sufficiently severe to warrant clinical attention.	E	Rapid onset and fluctuations of the symptoms over the course of the day.
		F	Objective evidence from history, physical and neurological examination or laboratory tests of an underlying cerebral or systemic disease (other than psychoactive substance-related) that can be presumed to be responsible for the clinical manifestations in A-D. Comments Emotional disturbances such as depression, anxiety or fear, irritability, euphoria, apathy or wondering perplexity, disturbances of perception (illusions or hallucinations, often visual) and transient delusions are typical but are not specific indications for the diagnosis.

communication among clinicians and healthcare professionals (Hall et al., 2012) and do not help promoting the knowledge of delirium. A recent statement of ten scientific societies and a review article proposed to update the nomenclature of delirium and acute encephalopathy, two commonly used terms to label the same phenomenon, to integrate them within a single framework, harmonize research efforts and advance clinical practice. According to this nomenclature, delirium should be considered a clinical syndrome while acute encephalopathy can be defined as a rapidly developing (usually within hours to a few days), diffuse pathobiological process that might manifest as delirium or, in cases of severely decreased levels of consciousness, as coma (Oldham and Holloway, 2020; Slooter and Stevens, 2020).

Another reason why delirium is undervalued is that, for many healthcare professionals, patients experiencing delirium may be only "agitated." It is possible that the concept of delirium somewhat recalls the condition known as "delirium tremens," which relates to alcohol abuse. But it is even more likely that the behaviors often associated with delirium, particularly agitation and aggression, causes distress and anxiety for nurses and families who then bring it to the attention of healthcare professionals. In a prospective Italian multicenter study where physicians diagnosed delirium according to their personal experience, delirium was coded in 2.9% of 2,521 older hospitalized patients, while combined deficits in attention, orientation, and memory (potentially suggestive of delirium) were found in 19.8% of patients (Bellelli et al., 2015b). Interestingly, the proportion of patients coded with delirium was similar to the proportion of patients who actually had hyperactive delirium in another Italian multicenter study, conducted on similar patients (Morandi et al., 2017). These findings suggest that, at least in Italy, physicians tend to diagnose delirium mainly when it is hyperactive. Thus, appropriate education and increasing broaden recognition of delirium syndrome is warranted.

The Challenging Diagnosis of Delirium Superimposed on Dementia

Delirium superimposed on dementia is a common condition in older people (Fick et al., 2002, 2013) but a real diagnostic challenge for clinicians, given the relative lack of tools specifically designed for its recognition. Indeed, the DSM-5 does not provide specific indications on how to diagnose delirium when overlapping on pre-existing dementia (American Psychiatric Association, 2013) and the CAM has shown only moderate sensitivity (77%) in detecting DSD (Morandi et al., 2012a). Other tools, such as the 4AT, have never been specifically tested in DSD patients (Jeong et al., 2020). The suboptimal performance of CAM and other existing tools to detect DSD mainly lies on the fact that people with dementia may already have impairments in cognitive functions, and especially in attention, which is a key feature of delirium (Marra et al., 2018). However, attention may be impaired in patients with severe dementia, limiting the ability of the attentional tests to discriminate between delirium and dementia (O'Halloran et al., 2014; Robertson et al., 2014; Bellelli et al., 2019). Thus, selecting the right testing is crucial. In 234 older patients admitted to an Emergency Department, Marra et al. found that MOTYB had very good sensitivities but had modest specificities for delirium, limiting their use as a standalone assessment, while reciting the days of the week backwards (DOWB) had the best combination of sensitivity and specificity (Marra et al., 2018). Nearly one third of their delirious patients had also dementia (Marra et al., 2018). In a prospective cohort study of patients admitted to an acute geriatric ward, Bellelli et al. (2019) assessed 89 patients, of whom 42 were frail and 29 had dementia. Patients were all assessed with three tests (i.e., the MOTYB, the DOWB and counting backwards from 20 to 1), which showed a similar predictive capacity to detect delirium in patients with frailty and dementia (Bellelli et al., 2019); further studies confirmed the association between frailty and attentional performances (O'Halloran et al., 2014; Robertson et al., 2014).

Recently, Steensma et al. (2019) proposed a brief screening test that included 3 items: (a) listing the days of the week, from Sunday to Monday, backwards; (b) asking the patient if he/she recognizes the type of place where he/she is currently located; and (c) report whether the patient appears sleepy. Among 391 older adults with dementia, they showed that the test had excellent sensitivity (94%) but limited specificity (42%) in recognizing DSD (Steensma et al., 2019).

Another key element to detect DSD is the patient's arousal, which is not usually impaired in dementia, even in the advanced stages. Therefore, in patients with impaired arousal (above the level of coma), the inability to engage in cognitive testing is considered severe inattention and thus a proxy of delirium (European Delirium Association and American Delirium Society, 2014). To evaluate the suitability of the tests assessing arousal for diagnosing DSD, a multicenter study recruited 645 patients with pre-existing dementia and measured their arousal's levels with the Richmond Agitation Screening Scale (RASS) and the modified RASS (m-RASS) (Ely et al., 2003; Chester et al., 2012). Overall, a score other than 0 at the two scales (i.e., the RASS and the m-RASS) was 70.5% sensitive and 84.8% specific for a diagnosis of DSD. Using a RASS/m-RASS value greater than + 1 or less than −1 as a cut-off, the sensitivity was 30.6% (CI 25.9–35.2%) and the specificity was 95.5% (CI 93.1−98.0%) (Morandi et al., 2016). The Observational Scale of Level of Alertness (OSLA) is another tool to evaluate the patient's arousal, and assesses patient's eye opening, eye contact, posture, movement, and communication showing to identify delirium specifically (Tieges et al., 2013). Recently, a European multicenter study recruited 114 patients with dementia alone, delirium alone, DSD or none, hypothesizing that a combined arousal and attention testing procedure would be accurate to detect DSD (Richardson et al., 2017). Using OSLA, 83% participants were correctly classified as having delirium while the attention test 76% of participants. However, combining scores correctly classified 91% of participants with delirium (sensitivity 84%, specificity 92%) and diagnostic accuracy remained high in the patients with dementia (sensitivity 94%, specificity 92%) (Richardson et al., 2017).

An additional observation is that delirium originating in a context of pre-existing cognitive impairment does not alter only the cognitive status but can affect the motor functions as

well (Bellelli et al., 2011; Gual et al., 2019). Indeed, one case-controlled study with prospective evaluations of four groups of patients with delirium alone, dementia alone, DSD or none, demonstrated that patients with delirium had fluctuations of motor performance (as assessed with the Trunk Control test) (Franchignoni et al., 1997) that were chronologically related with the onset of delirium, a pattern that was especially apparent in patients with DSD (Bellelli et al., 2011). Importantly, patients with DSD had severe pre-existing dementia (Bellelli et al., 2011). A more recent cross-sectional multicenter study recruiting 114 consecutive patients and measuring function with the Hierarchical Assessment of Balance and Mobility (HABAM) score (MacKnight and Rockwood, 1995), confirmed that individuals with delirium have worse motor function than those without delirium, especially if they had comorbid dementia (Gual et al., 2019). Pathophysiological explanations of these findings may include the acute imbalance of the cerebral brain networks connectivity that may occur during delirium (Maldonado, 2018).

We suggest that the assessment of motor functions can be helpful to detect DSD, especially in those patients with severe pre-existing dementia that are difficult to approach with verbal communication. A scheme for selecting the tools to assess delirium according to the severity of dementia and level of arousal is proposed in **Figure 2**.

PREVENTION

Non-pharmacological Approaches

There is robust evidence that non-pharmacological approaches are the best way to prevent delirium in hospitalized patients. In 1999, Inouye and colleagues described the hospital Elder Life Program (HELP), a model of care tailored for older patients and specifically oriented to prevent delirium during the hospital stay (Inouye et al., 1999). Eight hundred and fifty-two inpatients aged (≥70 years were enrolled, and the investigators carefully administered and tracked standardized interventions to manage six risk factors, assessed on admission. Those included vision impairment, hearing impairment, sleep deprivation, cognition, dehydration, and immobility. The intervention strategies included therapeutic activities, limited use of psychoactive drugs, reorientation, promotion of sleep, maintenance of adequate hydration and nutrition, early mobilization, and provision of visual and hearing adaptations. An experienced interdisciplinary team provides the HELP, with the assistance of trained nurses or volunteers. Overall, the authors found significant decrease in the incidence (number of episodes) and duration of delirium after applying this multicomponent program, suggesting that primary prevention may represent the most effective approach for treating this syndrome (Inouye et al., 1999). After this seminal work, the program has been replicated in other sites after being adapted (Inouye et al., 2000), and several systematic reviews and meta-analyses have assessed the efficacy of multicomponent non-pharmacological approach in preventing delirium. One systematic review and meta-analysis identified 14 high-quality trials for a total number of 4,267 patients, finding that a bundle of non-pharmacological and multicomponent interventions decreased the incidence of delirium by 44% (OR, 0.56; 95%CI, 0.42–0.76) (Hshieh et al., 2015). Importantly, this approach leads to a reduction of the rate of falls (Hshieh et al., 2015). Recent guidelines from Scientific Associations and Cochrane reviews incorporated these recommendations (Siddiqi et al., 2016; SIGN, 2019). The presence and involvement of family members at the patient's bedside has also shown potential efficacy in reducing the incidence of post-operative delirium and the rate of cognitive and physical deterioration at discharge (Wang et al., 2019). More recently, a systematic review integrated the evidence of the multicomponent interventions in ICU and non-ICU settings, confirming the current guidelines that non-pharmacological interventions are effective in preventing delirium, with a global risk ratio = 0.53 (95% CI = 0.41–0.69) (Ludolph et al., 2020).

Non-pharmacological approaches recommended by reviews and guidelines include the use of reorientation strategies (e.g., orientation boards, calendars, clocks), the promotion of patient's hydration, sleep, mobilization, and the use of assistive devices such as eyeglasses and hearing aids, if needed. Physical restraints should be avoided due to their role in worsening agitation and increasing risk of strangulation (Inouye et al., 2007). Another important element of non-pharmacological prevention includes the optimization of pain control with non-opioids and avoiding high-risk medications, such as those with anticholinergic effect (National Clinical Guideline Centre, 2010; SIGN, 2019).

Overall, despite strong evidence supporting their value, the implementation of delirium preventive measures is far from being the rule in most hospitals. Main barriers to implementation include the time constraints of the staff and cultural gaps that are widely diffused among physicians and nurses (Greysen, 2015). Furthermore, modifying the everyday practice in the acute hospital setting is difficult and is often perceived as risky and fraught with uncertainty, especially regarding the true benefits of the changing process for both patients and the healthcare system (Greysen, 2015). Future efforts are thus required to increase the rate of implementation of multicomponent non-pharmacological preventive measures of delirium, rather than pharmacological ones.

Pharmacological Approaches

The idea to prevent delirium using pharmacological interventions is fascinating but, at the present, poorly supported by the literature. Use of antipsychotics has been investigated, given their efficacy in psychiatric diseases. However, this approach is ineffective if not potentially harmful. Two recent systematic reviews evaluating the effect of first- and second-generation antipsychotics over placebo found no difference in delirium incidence, its duration, in the length of hospital stay, and in mortality among groups (Neufeld et al., 2016; Oh et al., 2019). Furthermore, some trials showed that the use of antipsychotics is associated with a higher occurrence of potentially detrimental cardiac effects (Oh et al., 2019). Benzodiazepines are similarly ineffective and may cause harm due to sedation, and should therefore be avoided, except in alcohol or benzodiazepine withdrawal-related delirium (SIGN, 2019).

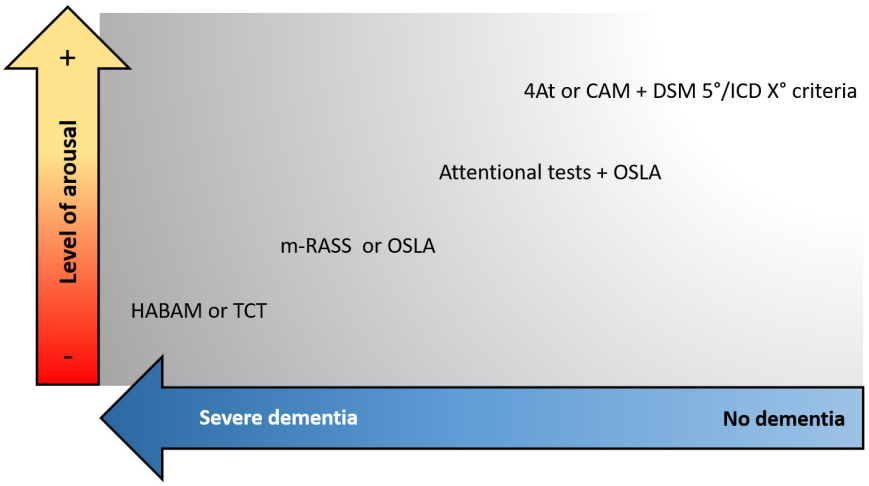

FIGURE 2 | Proposed approach to select the screening tools for delirium according to the presence of dementia and the patient's level of arousal. CAM, Confusion Assessment Method; DSM, Diagnostic and Statistical Manual of Mental Disorders; ICD, International Classification of Diseases; OSLA, Observational Scale of Level of Arousal; m-RASS, modified Richmond Agitation and Sedation Scale; HABAM, hierarchical assessment of balance and mobility; TCT, Trunk Control Test.

There may be some emerging promising evidence for melatonin and its endogenous hormone. A recent network meta-analysis of six randomized controlled trials demonstrated significant preventive effects with melatonin (both at 5 and 0.5 mg/day) and ramelteon (8 mg/day) against placebo groups (Yang et al., 2020). Furthermore, in a recent multicentre randomized placebo-controlled trial, Hatta et al. showed that suvorexant (used in the treatment of insomnia), every night for 3 days in 72 hospitalized older patients, significantly reduced the occurrence of delirium compared to the administration of placebo (Hatta et al., 2017). A meta-analysis that included seven studies conducted in patients undergoing this treatment compared with controls (402 treatment patients and 487 controls) showed that delirium incidence could be markedly reduced (Odds Ratio, 0.30; $P < 0.001$) and time to delirium onset was significantly lengthened in the treatment groups compared to controls (Xu et al., 2020). Further larger studies are required.

TREATMENT

Non-pharmacological Approaches

There are several guidelines from scientific and academic societies that offer guidance and practical recommendations in the management of delirium in older patients (National Clinical Guideline Centre, 2010; SIGN, 2019).

Once a patient is recognized with delirium, it is first important to identify acute and life-threatening causal factors, including hypotension, low tissue oxygenation, drug overdose or withdrawal, and hypoglycemia. Once life-threatening conditions have been corrected, the second step is the identification of the underlying causes of delirium. To help this task, some mnemonics have been proposed (Marcantonio, 2017). They may be particularly useful given that delirium has commonly a multiple etiology and the identification of only one cause may be insufficient, leading to inappropriate management of the patient (Ferrara et al., 2019). The third step is the adoption of non-pharmacological treatments. Unnecessary tubes, catheters and physical restraints should be avoided; conversely, approaching patients at the bedside with recognizable faces and adopting tools to facilitate orientation (e.g., calendar, clock) are useful to improve restlessness and mild-to-moderate agitation. It is also recommended to promote sleep hygiene and optimize nutritional and fluid intake (SIGN, 2019). Another potential target of treatment is the environment. Unfamiliar environment, excessive noise and ward moves may indeed be precipitating factors of delirium (Van Rompaey et al., 2012). It is also important to actively involve families (Rosa et al., 2017) and multidisciplinary professionals (such as occupational therapists) in care delivery, to improve mobility and participation (Pozzi et al., 2017).

Pharmacological Approaches

Delirium presenting with severe agitation (or combativeness) requires immediate non-verbal and verbal de-escalation techniques. If this approach fails, if agitation is severe and stressful for the patient and/or can endanger the provision of life-sustaining therapies, a pharmacological approach should be considered (National Clinical Guideline Centre, 2010). However, pharmacological agents result in sedation and may perpetuate delirium. Other steps must also be considered.

First, it is crucial to keep in mind that any changes in medications, including over-the-counter and herbal medications, or changes in dosage of medication or abrupt withdrawal of medication could result in delirium. Benzodiazepines, opiates, tricyclic antidepressants, anticholinergic medications, antihistamines and tramadol should be avoided or reduced if possible (SIGN, 2019). Constipation and urinary retention are common causes of severe agitation in people with moderate to severe dementia, and therefore, a systematic exclusion of

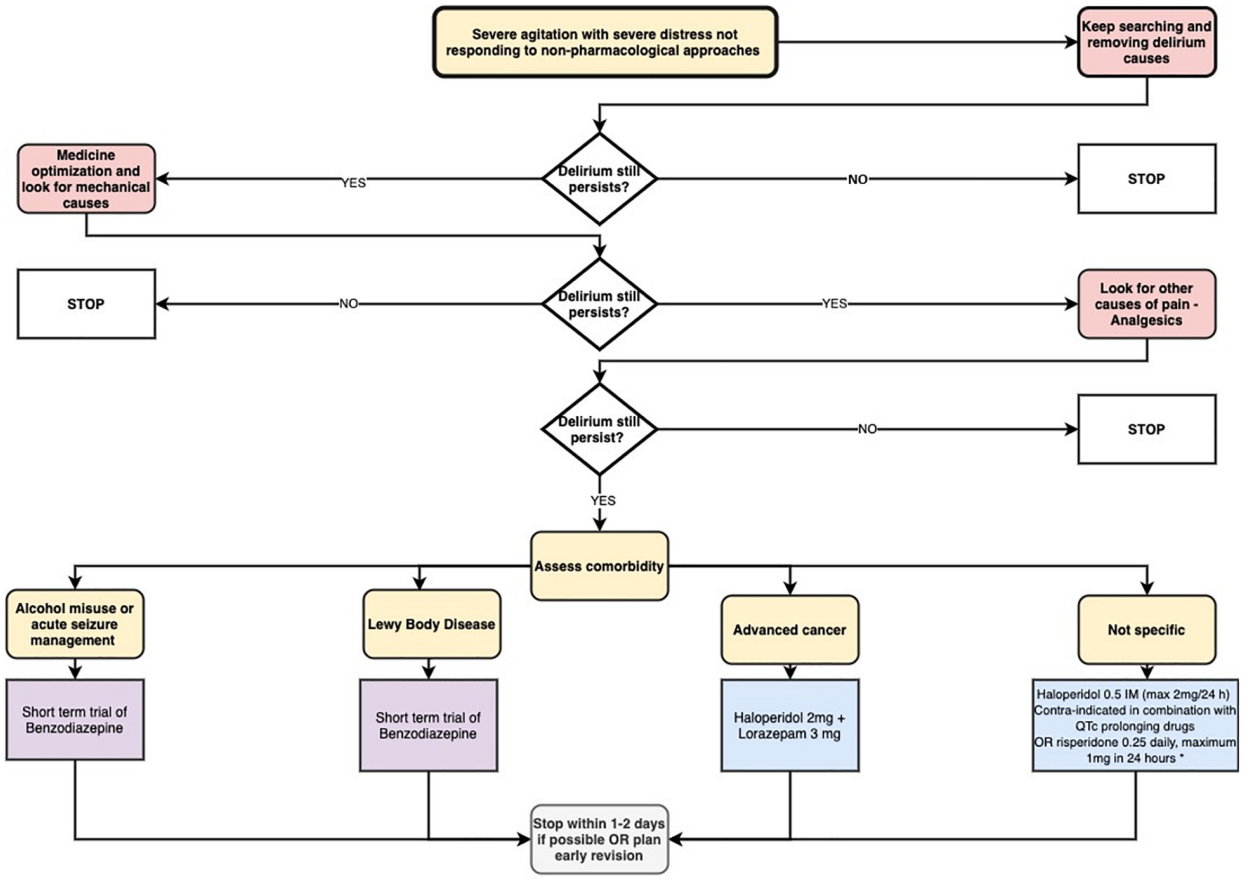

FIGURE 3 | Proposed pharmacological approach for the agitated delirium (Delirium: diagnosis, prevention, and management. NICE clinical guideline n. 103, 2010). * The single study supporting this indication was restricted to patients with advanced cancer in end-of-life care. Please note that this treatment option should be considered for refractory symptoms only (after antipsychotics have failed).

their presence is required. If pain is likely, e.g., due to trauma or painful procedures (e.g., surgery), or causes of delirium are not identified in a patient with moderate to severe dementia, analgesics should be initiated (Sampson et al., 2020). In fact, characteristic fluctuations in attention and awareness may affect the capability of self-reporting pain, making its recognition, assessment and treatment more difficult (Sampson et al., 2020). If analgesic treatment is required, non-opioid agents may be preferable, while the opioid with the lowest odds of causing delirium is oxycodone (SIGN, 2019).

The choice of antipsychotic medications in hyperactive delirium is object of debate, and recent systematic reviews have been published (Nikooie et al., 2019; Rivière et al., 2019). Rivière et al. investigated the efficacy and tolerability of atypical antipsychotics, finding some evidence that quetiapine and olanzapine are suitable alternatives to haloperidol (Rivière et al., 2019). However, the authors found high heterogeneity among studies that prevented them to perform a meta-analysis. Nikooie et al. extracted data from 16 randomized controlled trials and 10 observational studies of antipsychotic vs. another antipsychotic or antipsychotic vs. placebo, without finding differences and concluding that the routine administration of haloperidol or second-generation atypical antipsychotics is not supported by the current evidence (Nikooie et al., 2019). Again, the authors found wide heterogeneity in the dosage of antipsychotics, outcomes and measurement tools among studies (Nikooie et al., 2019).

A different approach is required if delirium is due to alcohol withdrawal, if there is a contraindication to antipsychotics (e.g., in neuroleptic malignant syndrome or Parkinson's disease), if the patient has dementia with Lewy bodies or for acute seizure management. In these cases, benzodiazepines should be considered (National Clinical Guideline Centre, 2010). Another case in which benzodiazepines may be used is the hyperactive delirium in patients with advanced cancer. One study performed among patients affected by advanced cancer focusing on the end-of-life period (last weeks or days of life), showed a reduction in agitated behavior by adding a single 3 mg dose of lorazepam intravenously to haloperidol, when compared with placebo (Hui et al., 2017). However, this treatment option should be considered for refractory symptoms only (after antipsychotics have failed), given that findings come from a small study, using a single dose of lorazepam.

Figure 3 summarizes the proposed pharmacological approach for the agitated delirium.

FUTURE PERSPECTIVES AND SUMMARY

As of now, the medical community still does not recognize delirium as it should, and this remains an international problem of immense gravity (Han et al., 2009; Erden Aki et al., 2014; Lange et al., 2019), which ultimately translates to improperly trained healthcare staff. Physicians may not actively search for the presence of delirium in their usual practice because they consider this task as superfluous and time expensive. On the contrary, we propose the systematic assessment of delirium as an opportunity for the clinicians to improve the clinical diagnostic process and thus the quality of care provided to their older patients. Here are reported the steps of this approach:

1. Because delirium is a frequent atypical presentation of diseases in the geriatric population, physicians should systematically screen it in their patients.
2. Given that delirium and its duration are associated with negative outcomes, delirium detection (and thus treatment) may be regarded as a clinical priority.
3. Once delirium has been detected, active searching for its underlying causes should be undertaken.
4. The tracking for the presence of delirium should be undertaken at least daily in acute hospital wards.
5. If, after treatment, delirium resolves, this may be an indirect sign that patient is clinically improving. Prolonged/persistent delirium may also be present despite the extensive diagnostic approach and therapeutic measures, indicating higher vulnerability or even a pathway to short-term or long-term cognitive impairment.

In other words, we propose to look at delirium as a marker of clinical instability and as a "*litmus test*" of the effectiveness of the care provided.

To summarize, delirium is a common and dangerous condition for older adults. Significant steps forward have been made regarding the understanding of pathophysiology and diagnosis, but still remain unresolved many aspects. Particularly challenging is the recognition and diagnosis of delirium superimposed on dementia. Prevention with multicomponent non-pharmacological approaches as well as non-pharmacological treatments are particularly important. Currently there are no drugs recommended for both prevention and treatment. Finally, the systematic detection of delirium is proposed as an opportunity for the clinicians to improve their clinical diagnostic process and thus the quality of care provided to their older patients.

AUTHOR CONTRIBUTIONS

GB, JB, and PM contributed to the conception and design of this work and drafted the manuscript. JB revised the manuscript critically for language and important intellectual content. All authors approved the final version of this work, and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity of any part of the work are appropriately investigated or resolved.

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Interactions Between Aging and Alzheimer's Disease on Structural Brain Networks

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Normative aging and Alzheimer's disease (AD) propagation alter anatomical connections among brain parcels. However, the interaction between the trajectories of age- and AD-linked alterations in the topology of the structural brain network is not well understood. In this study, diffusion-weighted magnetic resonance imaging (MRI) datasets of 139 subjects from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database were used to document their structural brain networks. The 139 participants consist of 45 normal controls (NCs), 37 with early mild cognitive impairment (EMCI), 27 with late mild cognitive impairment (LMCI), and 30 AD patients. All subjects were further divided into three subgroups based on their age (56–65, 66–75, and 71–85 years). After the structural connectivity networks were built using anatomically-constrained deterministic tractography, their global and nodal topological properties were estimated, including network efficiency, characteristic path length, transitivity, modularity coefficient, clustering coefficient, and betweenness. Statistical analyses were then performed on these metrics using linear regression, and one- and two-way ANOVA testing to examine group differences and interactions between aging and AD propagation. No significant interactions were found between aging and AD propagation in the global topological metrics (network efficiency, characteristic path length, transitivity, and modularity coefficient). However, nodal metrics (clustering coefficient and betweenness centrality) of some cortical parcels exhibited significant interactions between aging and AD propagation, with affected parcels including left superior temporal, right pars triangularis, and right precentral. The results collectively confirm the age-related deterioration of structural networks in MCI and AD patients, providing novel insight into the cross effects of aging and AD disorder on brain structural networks. Some early symptoms of AD may also be due to age-associated anatomic vulnerability interacting with early anatomic changes associated with AD.

Keywords: Alzheimer's disease, structural network, nerve fiber tracking, diffusion-weighted magnetic resonance imaging, cognitive impairment

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INTRODUCTION

Increasing evidence suggests that both aging and Alzheimer's disease (AD) can cause deterioration in anatomical brain connections, which is then associated with a decline in cognitive abilities (Peters, 2002; Perl, 2010; Teipel et al., 2016). Normal aging can undermine white matter organization, as nerve fiber loss increases with age. This decrease in the connections between distinct brain parcels contributes to a disruption in the normal flow of information through cortical networks (Betzel et al., 2014; Zhao et al., 2015; Wu et al., 2020). As a neurodegenerative disorder that reduces synaptic transmission (Morabito et al., 2015), AD also causes a gradual breakdown in brain structural connectivity, eventually resulting in dementia (Voevodskaya et al., 2018; Dai et al., 2019; Wu et al., 2019). This disruption of structural connectivity between key functional subregions may ultimately explain the characteristic deficits found in AD patients (Yao et al., 2010; Fischer et al., 2015; delEtoile and Adeli, 2017; Li et al., 2020). These age- and AD-related alterations in white matter organization can profoundly affect topological features of the brain structural network and synergistically damage its integrity (Palop et al., 2006).

Diffusion-weighted imaging (DWI) has often been employed to assess cerebral white matter tracts (Tuch et al., 2003; Sinke et al., 2018; Innocenti et al., 2019; Sotiropoulos and Zalesky, 2019). Pioneering studies have then used graph theory to quantify the brain structural organization, reporting meaningful results on brain networks in normal aging and AD (Yao et al., 2010; Stawarczyk et al., 2012; Ghanbari et al., 2014; Zhao et al., 2015). In particular, alterations in the topology of brain structural networks and their corresponding metrics reflect the regional interactions as they evolve in both normal aging and in AD progression. When used to address normative aging, decreased network efficiency has been demonstrated in hub regions, limiting their capacity to communicate (Gong et al., 2009; Zhao et al., 2015). This is believed to result from degeneration in the white matter microstructure (demyelination, Wallerian degeneration, gliosis, severe fiber loss, etc.; Burzynska et al., 2010; Damoiseaux, 2017; Reishofer et al., 2018) and contributes to lifelong decline (van den Heuvel and Sporns, 2013; Betzel et al., 2014; Gollo et al., 2018). For mild cognitive impairment (MCI) and AD, altered interregional correlations (particularly among the parahippocampal gyrus, medial temporal lobe, cingulum, fusiform, medial frontal lobe, and orbital frontal gyrus; Yao et al., 2010) lead to increased path lengths and decreased network efficiency (Lo et al., 2010; Fischer et al., 2015; delEtoile and Adeli, 2017), suggesting an impairment of structural networks in MCI and AD (He et al., 2008; Daianu et al., 2015; Raj et al., 2015). Especially, a structural *k*-core network analysis (examination of only nodes with a degree of *k* or higher) was performed on normal controls (NCs) and AD patients to investigate brain network breakdown as AD progresses (Daianu et al., 2013). This study found that white matter integrity deteriorated with age and was able to distinguish early MCI-linked white matter alterations from those that occurred during normal aging. The fact that aging and cognitive impairment could separately affect brain networks highlights the unique effects that each has on brain network topology. The interaction of these effects, however, has not yet been thoroughly addressed.

Considering that age effects are not restricted to healthy individuals, it is likely that the age-related disruption of structural networks can exacerbate the cognitive decline in MCI and AD patients. It is, therefore, necessary to recognize the distinct effects of aging and impairment on the brain structural networks, and how these separate factors can interact within both healthy individuals and those with MCI and AD. At the present time, age-related alterations in the structural networks of MCI and AD patients have not been comprehensively explored. In this study, the data from 139 subjects, obtained from Alzheimer's Disease Neuroimaging Initiative (ADNI) database (Jack et al., 2008) and divided into three age subgroups (56–65, 66–75, and 71–85 years), were used to assess the deterioration of structure that occurs with age. We included 45 NCs, 37 early MCI (EMCI), 27 late MCI (LMCI), and 30 AD patients. Statistical analysis focused on the cross effects between aging and AD progression on the topology of structural connectivity networks and investigated how the global and nodal topological metrics change with age, including network efficiency, characteristic path length, transitivity, modularity coefficient, clustering coefficient, and betweenness. From whole perspective, this study provides a complete view of AD-related topological changes in brain structural connectomes over time.

MATERIALS AND METHODS

Data

We used the ADNI database (adni.loni.usc.edu), launched in 2003 as a public-private partnership and led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether magnetic resonance imaging (MRI), positron emission tomography (PET), biomarkers, and clinical and neuropsychological assessment can be combined to measure the progression of MCI and early AD (Jack et al., 2008; Risacher et al., 2009; Petersen et al., 2010). In this study, 139 subjects aged from 56 to 85 years were selected from the ADNI database, including 45 NCs (32 females and 13 males), 37 EMCIs (18 females and 19 males), 27 LMCIs (11 females and 16 males), and 30 ADs (13 females and 17 males). The criteria for the classification of the subjects was based on mini-mental state examination (MMSE) and global clinical dementia rating (CDR) scores (Aisen et al., 2010). Whole-brain Diffusion-weighted imagings (DWIs) were collected from four MRI centers using the: (1) Siemens 3T scanner (7 b0 images, 48 DWIs with b = 1,000 s/mm^2, slice thickness = 2 mm, scanning sequence = EP, echo time = 0.056 s, repetition time = 7.2 s, flip angle = 90°); (2) the Siemens 3T scanner (13 b0 images, 48 DWIs with b = 1,000 s/mm^2, slice thickness = 2 mm, scanning sequence = EP, echo time = 0.071 s, repetition time = 3.4 s, flip angle = 90°); (3) the GE 3T scanner (6 b0 images, 48 DWIs with b = 1,000 s/mm^2, slice thickness = 2 mm, scanning sequence = EP_SE, echo time = 0.0606 s, repetition time = 7.8 s, flip angle = 90°); and, the (4) Philips 3T scanner (1 b0 image, 32 DWIs with b = 1,000 s/mm^2, slice thickness = 2 mm, scanning sequence = SE, echo time = 0.099 s, repetition time = 10.90 s, flip

angle = 90°; Daianu et al., 2013, 2015; Nir et al., 2015). ADNI data collection was performed after obtaining written informed consent from the participants. All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All DWI images were first denoised and corrected for eddy current and head movement distortions using MRtrix[1] and FSL[2] toolboxes. Then, DWI bias field correction was performed by estimating the bias field from b0 images. The whole flowchart of brain structural network construction by DWI is demonstrated in **Figure 1**.

Of note, there is still considerable controversy in the literature on the statistical analysis of structural network topology from multicenter DWI datasets (Tong et al., 2019). However, we suggest that topological characterization of structural networks would not significantly suffer from multicenter studies, as individual-based analysis of diffusion measures is not sensitive to the variability in MRI scanners (Tong et al., 2020). For the sake of reproducibility, the subject identifiers of each group are provided as Supplementary Materials.

Structural Network Construction

Brain structural networks can be represented as a graph, completely described by assigning a set of nodes and a set of edges with their corresponding weights (Hagmann et al., 2008; Garcés et al., 2016; Maier-Hein et al., 2017). In order to attain regional anatomical connectivity, the DKT template was applied to parcellate the whole brain into 62 subcortical regions (Potvin et al., 2017). **Figure 1D** demonstrates the DKT parcellation template, and **Table 1** lists the indices of regions of interests (ROIs). This template was co-registered into DWI native space to define ROIs for each subject. The MRtrix tool[1] was employed to reconstruct fiber tracks using deterministic tractography based on orientation distribution function (ODF) computed with constrained spherical deconvolution (CSD; Tournier et al., 2008). After fiber tracks were retrieved with the command "*tckgen -act*," spherical-deconvolution informed filtering of tractograms (SIFT) was employed to improve whole-brain streamlines reconstructions with the command "*tcksift*." Then, an inter-regional anatomical connectivity matrix was then obtained with "*tck2connectome -symmetric -zero_diagonal*," where the value of any element of the matrix is equal to the number of tracts originating in one region and terminating in (or passing to) another region. The number of fiber tracts between gray matter regions uncovered by MRtrix was determined from the data rather than defined *a priori*, and was therefore variable from individual to individual and from scan to scan (Bassett et al., 2011). Finally, the structural connectivity matrices were normalized into [0, 1] for topological characterization.

Topological Characterization

To characterize the underlying topological properties of brain structural networks, four commonly-used network-level and two nodal topological metrics were computed for each subject: efficiency, characteristic path length, transitivity, modularity coefficient, clustering coefficient, and betweenness centrality. These metrics were directly retrieved from structural connectivity matrices using the Brain Connectivity Toolbox[3] in MATLAB (The Mathworks, Inc., Natick, MA, USA; Rubinov and Sporns, 2010).

Network efficiency is a sensitive measure of network alterations that occur in aging and neurodegenerative disorders, which reflects the integration of information transfer within a given network. This effectively characterizes how well the information is communicated within the cerebral cortex and is expected to decrease with age (Gong et al., 2009). This metric is defined as:

$$E_{\mathrm{glob}}(G) = \frac{1}{N(N-1)} \sum_{i \neq j \in G} \frac{1}{L_{\mathrm{ij}}} \quad (1)$$

where L_{ij} is the shortest path length between node i and j in structural connectivity graph G. N denotes the number of nodes in the graph G.

The network characteristic path length is the average shortest path length between every pair of nodes in the network, which serves as a measure of overall network integration. This metric is inversely related to network efficiency (Cao et al., 2013) and quantifies the ability for information to be propagated in parallel. This metric was computed as:

$$L(G) = \frac{1}{N(N-1)} \sum_{i \neq j \in G} L_{\mathrm{ij}} \quad (2)$$

where L_{ij} is defined as the shortest path between node i and node j.

Transitivity measures the probability that the adjacent vertices of a vertex are connected, which is closely related to the clustering coefficient of a graph, as both measure the relative frequency of triangles (Rubinov and Sporns, 2010).

$$T(G) = \frac{3\lambda(G)}{\tau(G)} \quad (3)$$

where $\lambda(G)$ is the number of triangles in G, and $\tau(G)$ is total number of connected triples of nodes in G.

The optimal community structure is a subdivision of the network into nonoverlapping groups of nodes in a way that maximizes the number of within-group edges and minimizes the number of between-group edges. Modularity coefficient is a statistic that quantifies the degree to which the network may be subdivided into such clearly delineated groups. The modularity coefficient is defined as (Rubinov and Sporns, 2010):

$$Q(G) = \frac{1}{2m} \sum_{\mathrm{i,j}} \left[w_{\mathrm{i,j}} - \frac{k_{\mathrm{i}} k_{\mathrm{j}}}{2m} \right] \delta(c_{\mathrm{i}}, c_{\mathrm{j}}) \quad (4)$$

where $w_{\mathrm{i,j}}$ is the connection weight between node i and j. k_{i} and k_{j} are the sums of the weights of the edges attached to nodes i and j, respectively. m is the total link weight in the network overall. $\delta(c_{\mathrm{i}}, c_{\mathrm{j}})$ is 1 when nodes i and j are assigned to the same

[1]https://www.mrtrix.org
[2]https://fsl.fmrib.ox.ac.uk/
[3]http://www.brain-connectivity-toolbox.net/

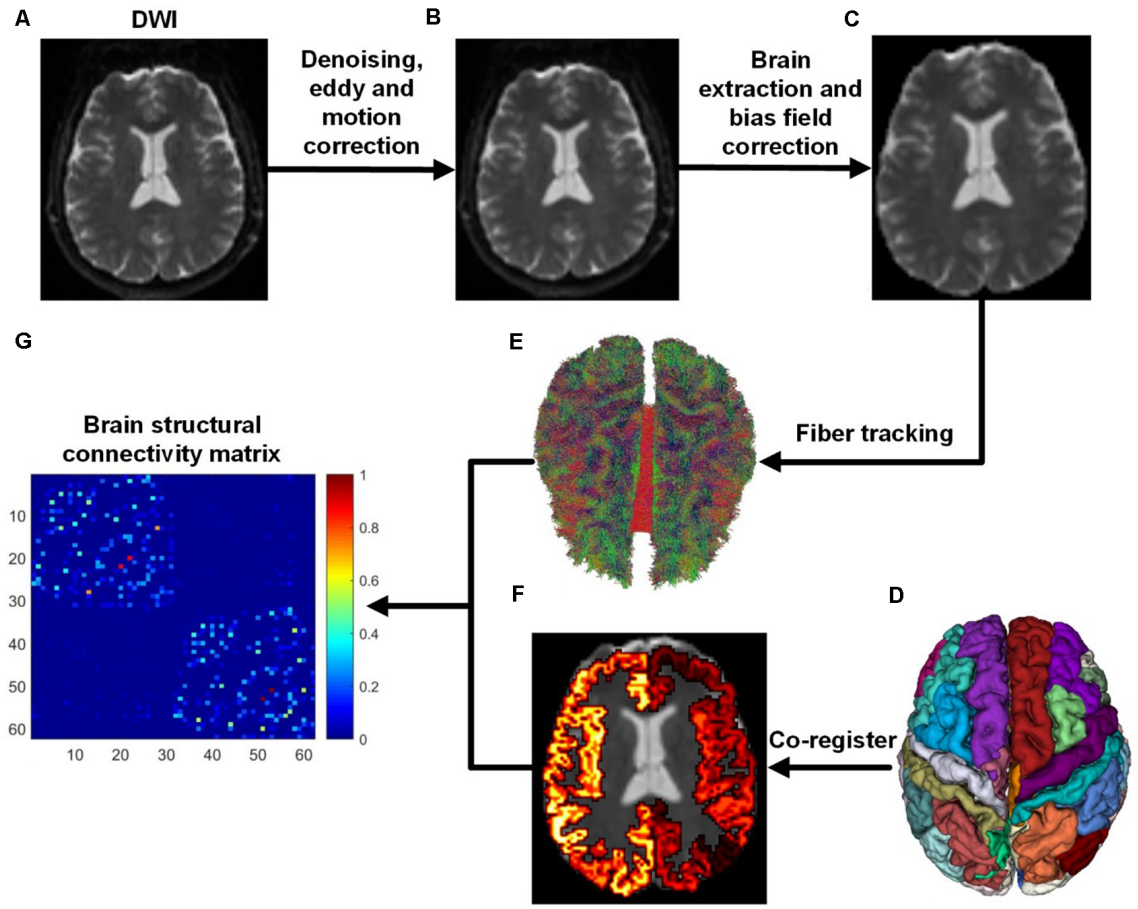

FIGURE 1 | Flowchart of brain structural network construction from diffusion-weighted imaging (DWI). Whole-brain models were parcellated into 62 different parcels according to the DKT template. **(A)** DWI. **(B)** After DWI was denoised, eddy and motion correction were performed. **(C)** Brain extraction and bias field correction. **(D)** DKT parcellation atlas. **(E)** White matter fibers reconstructed with anatomically-constrained tractography (Smith et al., 2012). **(F)** DKT atlas was co-registered into DWI native space. **(G)** Brain structural connectivity matrix was built by assigning fibers to each parcel.

TABLE 1 | Indexes of ROIs used to construct structural networks (Tzourio-Mazoyer et al., 2002).

Region	Region	Region	Region	Region
1 Left caudal anterior cingulate	**14** Left parahippocampal	**27** Left superior parietal	**40** Right lateral occipital	**53** Right precentral
2 Left caudal middle frontal	**15** Left paracentral	**28** Left superior temporal	**41** Right lateral orbitofrontal	**54** Right precuneus
3 Left cuneus	**16** Left pars opercularis	**29** Left supramarginal	**42** Right lingual	**55** Right rostral anterior cingulate
4 Left entorhinal	**17** Left pars orbitalis	**30** Left transverse temporal	**43** Right medial orbitofrontal	**56** Right rostral middle frontal
5 Left fusiform	**18** Left pars triangularis	**31** Left insula	**44** Right middle temporal	**57** Right superior frontal
6 Left inferior parietal	**19** Left pericalcarine	**32** Right caudal anterior cingulate	**45** Right parahippocampal	**58** Right superior parietal
7 Left inferior temporal	**20** Left postcentral	**33** Right caudal middle frontal	**46** Right paracentral	**59** Right superior temporal
8 Left isthmus cingulate	**21** Left posterior cingulate	**34** Right cuneus	**47** Right pars opercularis	**60** Right supramarginal
9 Left lateral occipital	**22** Left precentral	**35** Right entorhinal	**48** Right pars orbitalis	**61** Right transverse tempora
10 Left lateral orbitofrontal	**23** Left precuneus	**36** Right fusiform	**49** Right pars triangularis	**62** Right insula
11 Left lingual	**24** Left rostral anterior cingulate	**37** Right inferior parietal	**50** Right pericalcarine	
12 Left medial orbitofrontal	**25** Left rostral middle frontal	**38** Right inferior temporal	**51** Right postcentral	
13 Left middle temporal	**26** Left superior frontal	**39** Right isthmus cingulate	**52** Right posterior cingulate	

module and 0 otherwise. Larger Q values are indicative of a highly modular network organization, while lower Q values indicate a more uniform network structure.

To assess the effect of aging and AD progression on local brain regions, node clustering coefficient and betweenness centrality were estimated for each group. The weighted clustering

coefficient is the average intensity of all triangles associated with each node, which indicates the extent of local interconnectivity or cliquishness in a network (Daianu et al., 2013; Otte et al., 2015).

$$c_i = \frac{2}{k_i(k_i-1)}\sum_{j,k}(w_{i,j}w_{j,k}w_{k,i})^{1/3} \quad (5)$$

where k_i is the degree of node i, and w denotes the structural connection weight.

Node betweenness centrality is the number of shortest paths that pass through a node (Equation 5). High betweenness centrality values indicate more passages traversing a node. In this work, betweenness centrality was normalized to the range [0, 1] as betweenness/$[(N-1)(N-2)]$ (Rubinov and Sporns, 2010).

$$b_i = \sum_{h\neq j, h\neq i, j\neq i}\frac{p_{hj}(i)}{p_{hj}} \quad (6)$$

where p_{hj} is the number of shortest paths between nodes h and j, and $p_{hj}(i)$ is the number of shortest paths between h and j that pass through the node i.

Statistical Analysis

The objective of this study is to assess the interactive effects of aging and AD progression on topological properties of the brain structural network. After the gender covariate was regressed out, linear regression and ANOVAs were adopted for statistical analysis and performed. In order to estimate the changing trajectories of topological measures over age, linear regression was separately performed on each global metric in the NC, EMCI, LMCI, and AD groups, respectively. To test whether the network-level topology of structural networks was significantly different over age and across NC, EMCI, LMCI, and AD groups, group-wise comparisons of network-level topological measures were performed using one-way ANOVA tests. Finally, to characterize the interaction between aging and AD progression on network-level and nodal topological properties, two-way ANOVA tests with the two factors of age and AD propagation stage were employed to identify group-wise differences. The factor of age consists of three levels: 56–65 years, 66–75 years, and 76–85 years. And the factor of the AD stage includes four levels: NC, EMCI, LMCI, and AD. A significance level of p-value <0.05 (uncorrected) was used for ANOVA tests.

RESULTS

Linear Regression on Network-Level Topological Metrics

Linear regression was performed on the global topological metrics (network efficiency, characteristic path length, transitivity, and modularity coefficient) to examine whether, over age, the structural networks of MCI and AD patients exhibited similar deterioration patterns. **Figure 2** shows the results, which indicate that the characteristic path lengths (Slope: 0.21, 0.12, 0.18, 0.05) of the NC, EMCI, LMCI, and AD groups increased with age, while the metric of efficiency (Slope: −0.0004, −0.0002, −0.0006, −0.0002) decreased. However, except for the EMCI group, the transitivity of the NC, LMCI, and AD groups were nearly unchanged. While modularity coefficients (Slope: 0.0010, 0.0008, 0.0002) of NC, EMCI, LMCI groups increase with age, the coefficient of the AD group remained unchanged. R-square values of the linear regression were present on the left corner of each subgraph. Overall, linear regression results indicated that the integrity of the structural networks of NC, MCI, and AD individuals all roughly deteriorated with age. However, lesser age-related effects were found in the metrics of the AD group.

ANOVA Tests on Topological Measures

Differences in the global topological measures between the three age subgroups (56–65, 66–75, and 71–85 years) were assessed using one-way ANOVA tests. **Figure 3** demonstrates the comparison results, and the asterisk sign (*) indicates that p-value <0.05 (uncorrected). For the NC group, differences between age subgroups in network efficiency and characteristic path were statistically significant (p-value = 0.0116 and p-value = 0.0134, respectively). For EMCI, differences in efficiency, characteristic path and clustering coefficient were significant (p-value = 0.0467, p-value = 0.0256 and p-value = 0.0069, respectively). For LMCI subjects, differences between age groups in efficiency and clustering coefficient were significant (p-value = 0.0211 and p-value = 0.0315, respectively). No metrics significantly differentiated the two age subgroups within the AD group.

Additionally, to detect group-wise differences among NC, EMCI, LMCI, and AD subjects, one-way ANOVA tests were also carried out. Results are shown in **Figure 4**, and pairwise groups that exhibited significant differences were identified and marked with an asterisk sign (*), indicating that p-value <0.05 (uncorrected). For the three age groups, efficiency and characteristic paths do not significantly distinguish the NC, EMCI, LMCI, and AD groups. However, for the three age groups, significant differences were only found in the metric of the modularity coefficient. Interestingly, a significant difference between the NCs and LMCI groups was only found in the 56–65 years group. This may be attributed to individual variability. In summary, while the modularity coefficient was most sensitive to AD propagation across 56–75 years, no significant difference was identified in terms of this metric among NC, LMCI, and AD subjects in the 76–85 years group.

Two-way ANOVA tests were also separately performed on the global topological metrics, and the combined changing trajectories of mean values of these metrics are shown in **Figure 5**. In summary, no significant interactions were found between aging and AD propagation in terms of these network-level metrics.

To reveal the interactive effect of aging and AD progression on local topological properties, nodal clustering coefficient and betweenness centrality were estimated for each subject. Using two-way ANOVA tests, it was found that multiple regions, including the left lateral occipital (9), left postcentral (20), right caudal anterior cingulate (32), right inferior parietal (37), right rostral anterior cingulate (55), and right superior frontal (57) exhibited significant differences in terms of clustering coefficient over age (**Table 2** and **Figure 6**). Moreover, significant differences were found only in the parcel of right insula

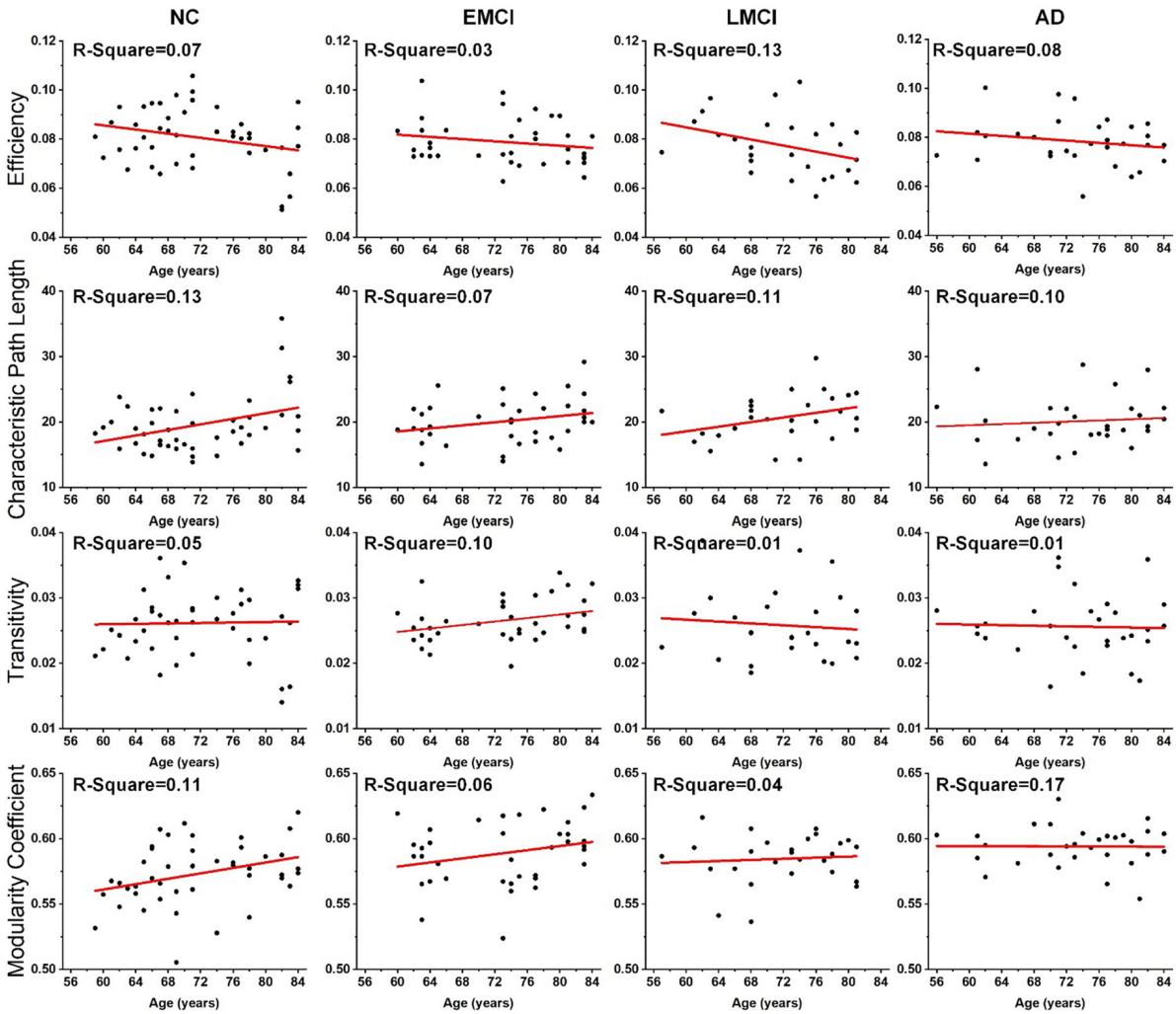

FIGURE 2 | Age effects on the global topological properties of structural networks, including global efficiency, characteristic path length, transitivity, and modularity coefficient. *R*-square value is present on the left corner of each subgraph. The fitted lines are shown in red, and the black dots represent the metric values of each subject.

(62) across AD propagation stages age (**Table 2** and **Figure 6**). Regional betweenness centrality values in the left entorhinal (4), left fusiform (5), left middle temporal (13), left posterior cingulate (21), right lingual (42), right precentral (53), right rostral anterior cingulate (55), and right superior frontal (57), showed significant differences across age subgroups (**Table 2** and **Figure 6**). In addition, the left rostral anterior cingulate (24) and right insula (62) parcels were identified to have significant differences across AD stages in terms of betweenness (**Table 2** and **Figure 6**). Finally, for clustering coefficient, the cortical parcels of right pars triangularis (49) and right precentral (53) exhibit significant interaction between aging and AD propagation stages (**Table 2** and **Figure 7A**). For the metric of betweenness, significant interactions between aging and AD stages were found in the left superior temporal (28) and right pars triangularis (49) (**Table 2** and **Figure 7A**). The cortical parcels that exhibited significant groupwise differences and interactions are summarized in **Table 2**, and the corresponding positions of these parcels are displayed in **Figures 6**, **7A**.

DISCUSSION

Cortical connectivity can be seen to reduce with age and AD progression, leading to significant deficits in topological properties of the structural network. These topological metrics provide valuable insights into the deteriorating neurological processes underlying aging and AD progression, offering a unique way to evaluate the impairment of anatomical connectivity patterns. In this study, we constructed brain structural networks of NC, EMCI, LMCI, and AD subjects by calculating fiber bundle numbers between pairs of gray matter parcels and investigating the cross effects of aging and AD progression on network-level and nodal structural topography. The results confirm that normal aging and AD propagation could

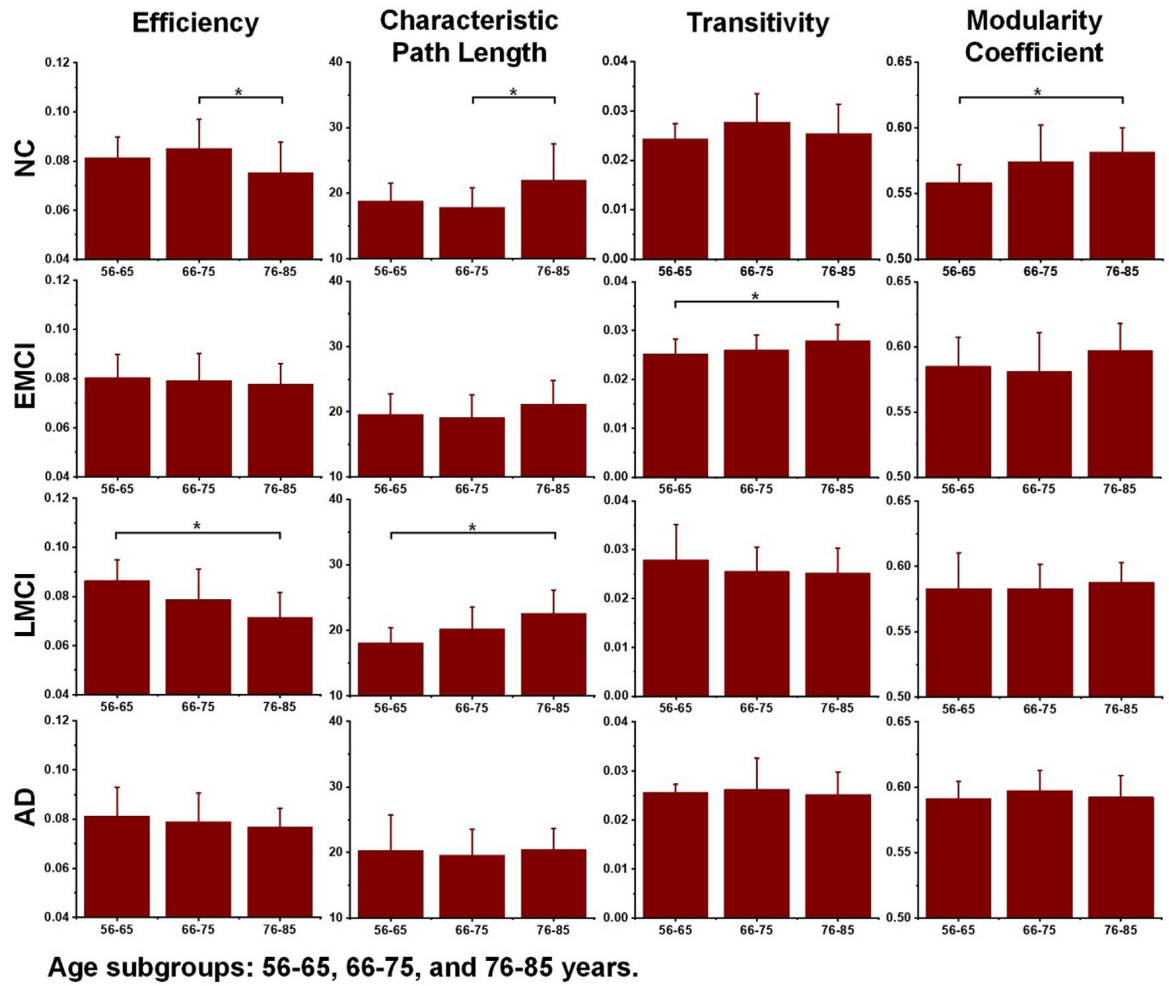

FIGURE 3 | Differences between age subgroups (56–65, 66–75, and 76–85 years) in network efficiency, characteristic path length, transitivity, and modularity coefficient. Group differences were estimated using one-way ANOVA, and the asterisk sign (*) indicates that p-value < 0.05 (uncorrected).

both affect the integrity of brain structural networks, and indicate that the network-level metrics of AD structural networks were relatively more deteriorated than those of NCs. Overall, however, more significant age-related differences were indicated in healthy controls than in AD patients.

Several recent studies based on DWI have demonstrated that the efficiency of structural networks decreases during normal aging due to neuronal shrinkage, loss of axon fibers, and whiter matter degeneration (Gong et al., 2009; Zhao et al., 2015; Sheffield et al., 2019). To reveal age-related degeneration in the white matter microstructure of NCs, MCI, and AD, this study performed linear regression on each of the global topological metrics, separately. Results provided new insight into the age-related changes in brain structural networks of healthy, MCI, and AD individuals, which are crucial for understanding how age affects the structural connectome of AD disorders. For all groups, network efficiency decreased with increasing age while characteristic path length increased. This is in accordance with previous studies (Meunier et al., 2009; Betzel et al., 2014; Fischer et al., 2015; Zhao et al., 2015). As shown in **Figure 2**, the deteriorated network-level topological properties of brain structural networks found in this study may provide the underlying substrate for the functional decline observed in aging individuals. In terms of the metrics of efficiency, characteristic path length, and modularity coefficient, the age-related deterioration in structural networks of AD patients is less significant than for healthy and older adults with MCI. We may infer that the anatomical connectivity breakdown caused by AD weakens the detrimental effect of aging on brain structural networks. Additionally, no significant age-related differences were identified in the AD subgroup (**Figure 3**), weighing against the hypothesis that aging leads to a vulnerability to the spread of AD.

AD progression can be characterized by a loss of connected areas in terms of global topological measures including network efficiency, characteristic path length, transitivity, and modularity coefficient. Much evidence from previous studies supports the interpretation of AD as a disconnection syndrome

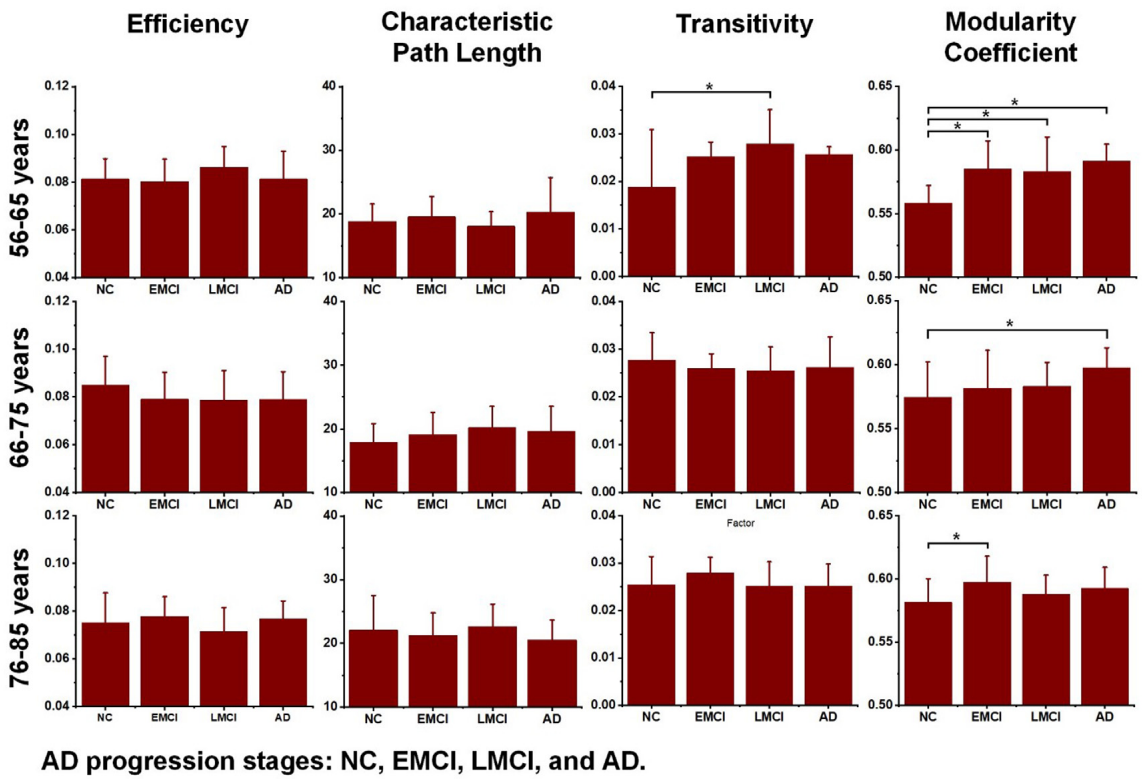

FIGURE 4 | Differences across the normal control (NC), early mild cognitive impairment (EMCI), late mild cognitive impairment (LMCI), and Alzheimer's disease (AD) groups were estimated using one-way ANOVA tests in terms of network efficiency, characteristic path length, transitivity, and modularity coefficient. The asterisk sign (*) indicates that the p-value was < 0.05 (uncorrected).

(Fischer et al., 2015; Morabito et al., 2015; Guo et al., 2016). To further reveal how AD propagation affects structural networks, one-way ANOVA tests were employed to explore groupwise differences across the NC, EMCI, LMCI, and AD groups in each age subgroup (56–65, 66–75, and 76–85 years). **Figure 4** demonstrates that in the 56–65- and 66–75-years age groups, there were significant differences between NCs and ADs in terms of modularity coefficient. For the 75–85 years group, however, no significant difference in modularity coefficient was detected, indicating that aging and AD both lead to inter-module disconnection in brain structural networks. Interestingly, as shown in **Figure 4**, the other global metrics (network efficiency, characteristic path length, and transitivity) did not exhibit significant differences between NC and AD subjects in the three age groups (p-value > 0.05). The reasons why there is no difference between the groups regarding efficiency and path length are manifold. This work is based on cross-sectional ADNI data. There are individual variabilities in brain structural networks. The progression stages of AD are generally defined by MMSE and CDR test scores. At present, it's unclear if the subjects with the same test score share the same brain structural networks. Another hypothesis could be that neural plasticity would alter structural connectivity during AD progression. Some subjects may have better neural plasticity than others. Hence, their connectivity could be better or worse than predicted. These one-way ANOVA tests indicate that the AD subjects are associated with greater structural connectivity deterioration in younger adults, while cognitive impairments have relatively lesser effects on older adults. Age-related alterations of whole-brain white matter network properties of AD patients were not detectable. However, the underlying neurophysiological reasons may be worthy of further study.

To comprehensively reveal the interaction between aging and AD progression on brain structural networks, using two-way ANOVA tests, the cross effect of aging and AD progression on local topological properties has been assessed in terms of node clustering coefficients and betweenness centrality (**Table 2**, **Figures 6**, **7**). The results indicate that aging and AD progression interactively and significantly affect some local regions, including the left superior temporal, right pars triangularis, and right precentral. This may occur due to broken anatomical connections between these cortical subregions and others, which were interactively affected by aging and AD progression. The three parcels are related to language understanding and motor movement (Foundas et al., 1996; Yousry et al., 1997; Aeby et al., 2013), and these cognitive functions both gradually deteriorate with age and AD progression. Previous studies have found that subjects with MCI and AD have a significant reduction in structural connectivity in the superior temporal lobe, medial temporal

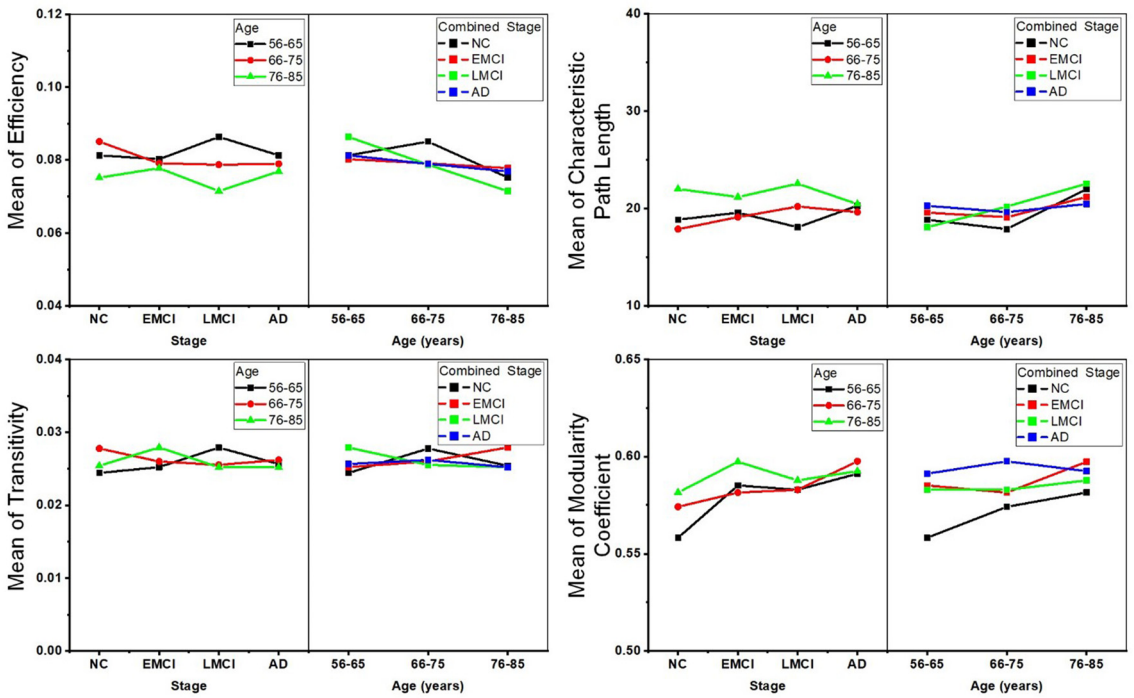

FIGURE 5 | Combined changing trajectories of the four network-level topological metrics (efficiency, characteristic path length, transitivity, and modularity coefficient). No significant interactions were found between aging and AD propagation at the level of p-value < 0.05 (uncorrected).

TABLE 2 | The cortical subregions with significant differences were identified in terms of clustering coefficient and betweenness centrality.

Nodal metrics	The population means of Age are significantly different (p < 0.5)	The population means of Stage are significantly different (p < 0.5)	The interaction between Age and Stage is significant (p < 0.5)
Clustering Coefficient	left lateral occipital (9), left postcentral (20), right caudal anterior cingulate (32), right inferior parietal (37), right rostral anterior cingulate (55), right superior frontal (57)	right insula (62)	right pars triangularis (49), right precentral (53)
Betweenness	left entorhinal (4), left fusiform (5), left middle temporal (13), left posterior cingulate (21), right lingual (42), right precentral (53), right rostral anterior cingulate (55), right superior frontal (57)	left rostral anterior cingulate (24), right insula (62)	left superior temporal (28), right pars triangularis (49)

*The listed brain parcels in this table were visualized on Montreal Neurologic Institute 152 (MNI152) brain images, as shown in **Figures 6**, **7A**. The numbers in parentheses were the indexes of cortical parcels (**Table 1**). The p-values were not corrected.*

lobe, inferior parietal areas, and lingual gyri (Bell-McGinty et al., 2005; Yao et al., 2010; Zhao et al., 2015). Age-related structural network studies also revealed that regional efficiency reduced in the parietal and occipital lobes with age (Gong et al., 2009; Burzynska et al., 2010; Zhao et al., 2015). To some extent, our result is in accordance with these prior studies. Specifically, no significant group-wise difference or interaction was found in the occipital area and hippocampus subregions (14 and 45) in this study. A possible reason for this may be the choices of whole-brain parcellation atlas and nodal metrics. Additionally, as the most serious hippocampal pathology may be already present when the diagnosis of MCI or AD was made, hippocampal connections could not have much additional deteriorations over time. The present results are, to some extent, consistent with these studies: cognitive function deficits could be due to abnormalities in the connectivity between these brain areas. This region-specific topological analysis provides insight into the aberrant topological patterns induced by interaction between aging and AD propagation.

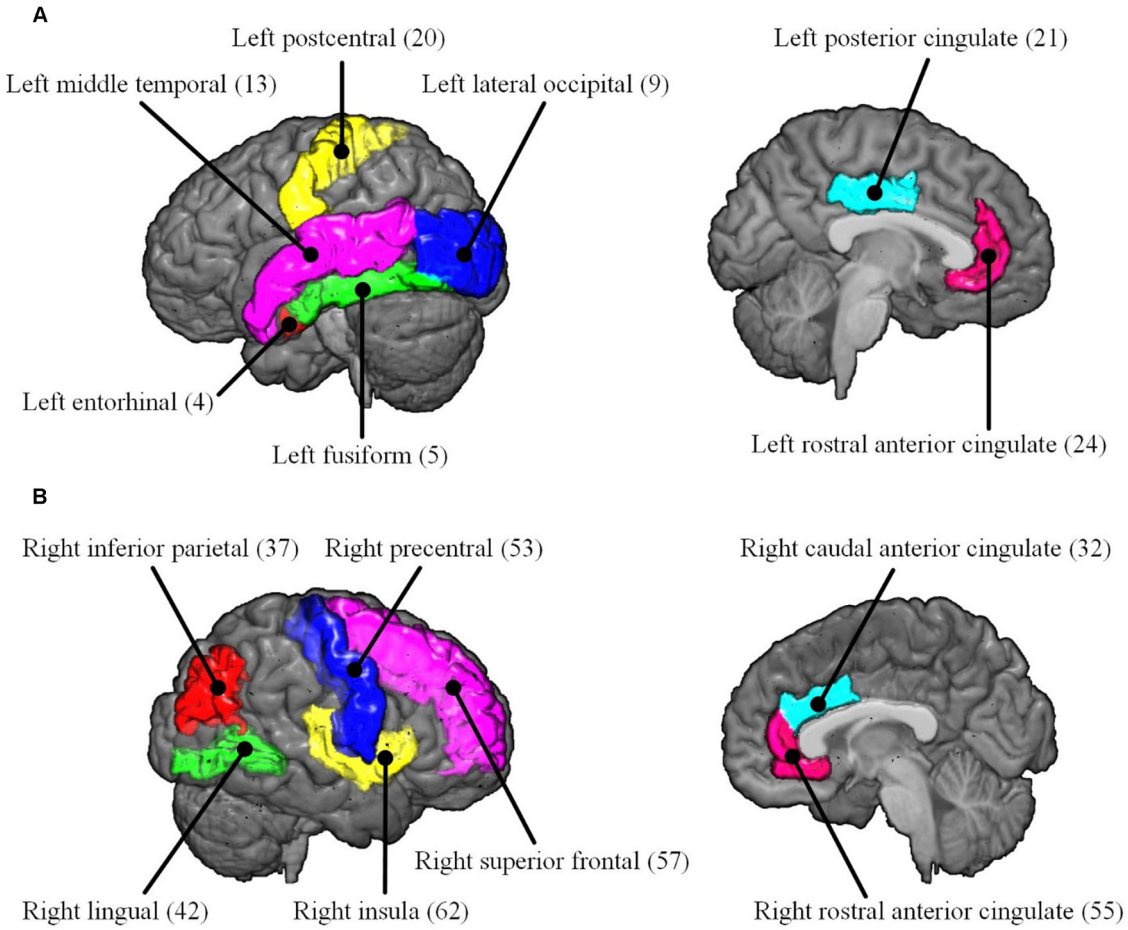

FIGURE 6 | The cortical parcels listed in **Table 2** are visualized on MNI152 brain images. The numbers in parentheses are the indexes of parcels (**Table 1**). **(A)** Left hemisphere. **(B)** Right hemisphere.

Several methodological issues about this study should be addressed. First, the DKT atlas was used to parcellate the whole cortex. When different parcellation schemes are used to define network nodes, topological metrics may be different (Wu et al., 2019). Second, the edges of the white matter networks were reconstructed by deterministic tractography based on CSD. Future studies should employ more advanced tractography techniques, such as probabilistic tractography to define the network edge weights (Sotiropoulos and Zalesky, 2019). Third, to ascertain the real structural networks as accurately as possible, this study included as many subjects as were available from each group in the ADNI database, which made the sample sizes of each group inconsistent. Fourth, as the DWI datasets are collected from multiple MRI centers, network consistency still needs to be confirmed. For different patients, AD onsets may start in distinct brain areas (Ossenkoppele et al., 2020), and this may influence the statistical analysis of local topological characterization. Finally, as the cause for white matter hyperintensities remain uncertain (Merio, 2019), we did not consider this factor in the statistical tests. Interaction across aging, AD progression, and neural plasticity (Bernhardi et al., 2017) complicates the analysis of brain structural connectivity deterioration due to AD. In the future, the combination of the multimodal MRI techniques (structural, diffusion-weighted, and functional MRI) should yield a comprehensive understanding of the relationship between structural and functional changes during normal aging and AD progression.

CONCLUSION

Brain network analysis offers a promising new approach to track and understand aging and AD progression. From this study, we conclude that age-related deterioration in structural networks contributes less to AD patients than healthy old adults. While no significant interaction is identified between aging and AD propagation in terms of the network-level metrics, significant interaction is found in the parcels of left superior temporal, right pars triangularis, and right precentral in terms of nodal clustering coefficient and betweenness. These findings may explain how network abnormalities in AD patients gradually evolve over time. In summary, our results emphasize age- and AD-related degeneration of specific brain parcels, thus providing novel

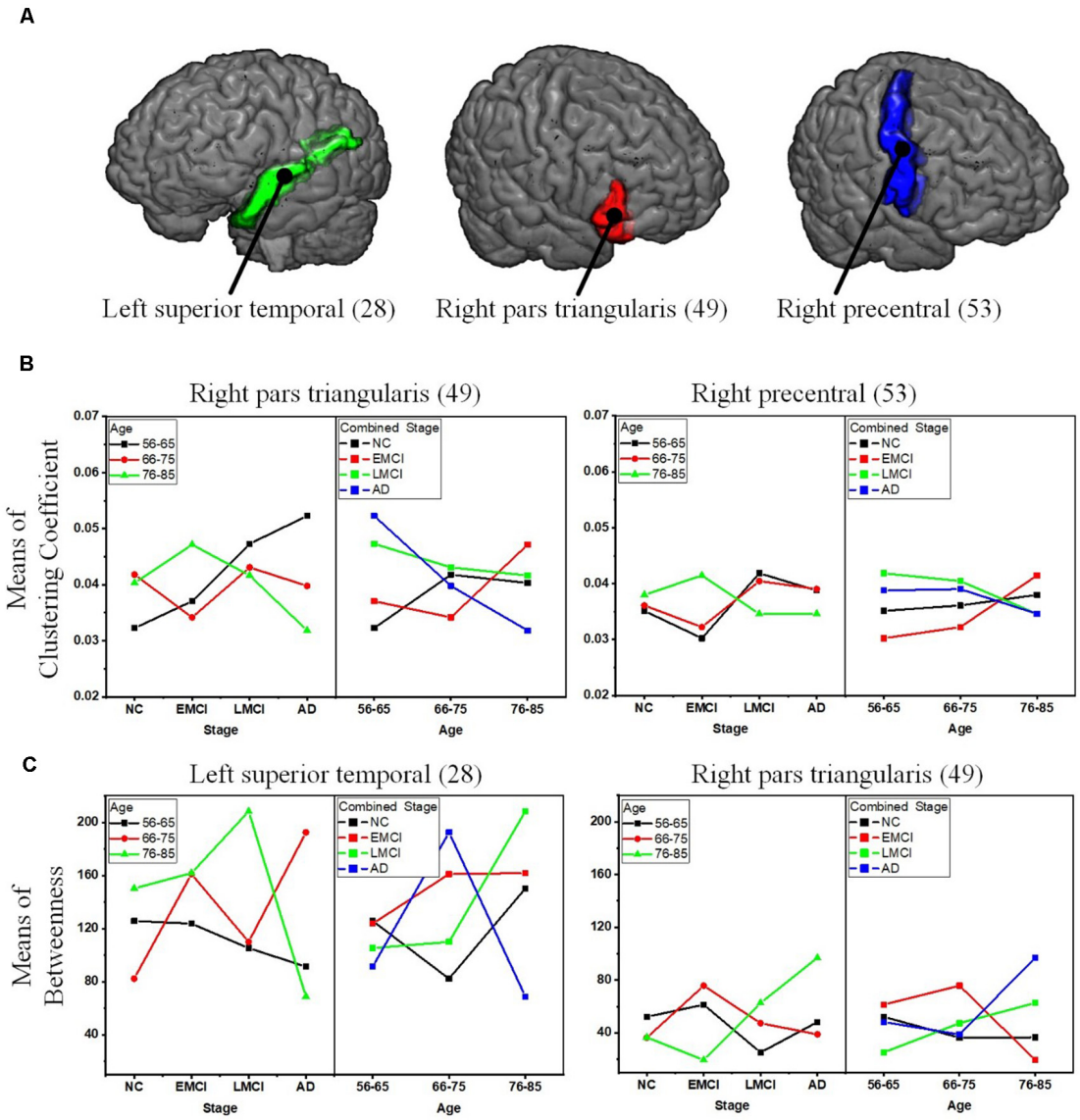

FIGURE 7 | Significant interactions between aging and AD propagation were found in the cortical parcels of left superior temporal, right pars triangularis, and right precentral. **(A)** Visualization of the parcels of left superior temporal, right pars triangularis, and right precentral. **(B)** Combined changing trajectories of mean values of clustering coefficient. **(C)** Combined changing trajectories of mean values of betweenness.

insights into the underlying pathophysiological mechanisms of connectivity alterations over aging and AD progression. This also indicates the potential of using these parcels' topological metrics as a diagnostic biomarker. Further studies for neurophysiological correlation between aging and AD progress are still needed to comprehensively assess their cross effects on the integrity of structural connectivity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ZW analyzed and interpreted the data and was a major contributor in writing the manuscript. YG assisted in analyzing data and interpreting the results, and also contributed to

manuscript writing. TP assisted in interpretation of the results and manuscript writing. JB performed the statistical analysis and assisted in manuscript writing. JS assisted in analyzing and interpreting the data, and also contributed to manuscript writing. PS assisted in the result interpretation and manuscript writing and revision. YZ supervised the design of study, interpreted results, and contributed to manuscript writing. All authors contributed to the article and approved the submitted version.

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Information-Theoretic Quantification of Dedifferentiation in the Aging of Motor and Executive Functions

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A central account of cognitive aging is the dedifferentiation among functions due to reduced processing resources. Previous reports contrasting trends of aging across cognitive domains mostly relied on transformed scores of heterogeneous measures. By quantifying the computational load with information entropy in tasks probing motor and executive functions, this study uncovered interaction among age, task, and load as well as associations among the parametric estimates of these factors at the individual level. Specifically, the linear functions between computational load and performance time differed significantly between motor and executive tasks in the young group but not in the elderly group and showed stronger associations for parameters within and between tasks in the elderly group than in the young group. These findings are in line with the dedifferentiation hypothesis of cognitive aging and provide a more principled approach in contrasting trends of cognitive aging across different domains from the information-theoretic perspective.

Keywords: Fitts' law, majority function, BIT, generalized linear mixed models, dedifferentiation

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INTRODUCTION

Aging impacts cognitive functions in a variety of manners: while some functions have been found to deteriorate (e.g., fluid intelligence), others seem to remain relatively stable across years of later adulthood (e.g., crystallized intelligence; Park and Gutchess, 2002; Salthouse, 2019). As the population worldwide rapidly grows older, precisely depicting trajectories of aging for different cognitive functions have great values from various perspectives, including clinical applications, policymaking, life span education, industrial research, and development, etc. At the group level, both cross-sectional and longitudinal reports comparing standardized scores of cognitive functions across life span suggest distinctive progression of aging-related impacts on different cognitive domains (Park and Gutchess, 2002; Salthouse, 2019). Outlining the landscape of aging across multiple cognitive domains is not a trivial business, considering the wild variety of the ways that different cognitive functions within a domain are operationally defined, measured, and compared. This study aims to examine this landscape by exploring the difference and relationship between two cognitive domains that are known to be susceptible to the impacts of aging, namely, the executive, and motor control.

As cognition comprises a vast array of diverse functions and researchers having accumulated numerous paradigms in studying them, it is almost unavoidable to apply a certain scheme of standardization when constructing any panoramic view of cognitive aging. For example, in the most widely applied procedure, *z*-standardization, the observations are demeaned, and divided by the sample SD. In the context of cognitive aging, the standardized performance indices of

different abilities as functions of age are then contrasted for assessing the trend. As one loses the absolute scale of the performance measure, the scope of interpretation from the *z*-score approach is limited to the comparison of general trends in the ability of each individual. Further exploration of the relationships among trends in different abilities can be misleading, due to the fact that standardization often distorts the distances between observations and the multivariate distributions of cross-sectional (Fischer and Milfont, 2010) and longitudinal data (Moeller, 2015). While alternative ways of transforming heterogeneous measures have been proposed to overcome the above problems (Little, 2013), a fundamental resolution is to design tasks for different domains in ways that independent variables vary along a commensurable dimension. Specifically, this is viable by applying the information theory (Shannon and Weaver, 1949) in quantifying the computational load involved in various cognitive functions (Fan, 2014).

Although the information theory sits at the core of the "cognitive revolution" (Neisser, 1976), in practice, it served mostly as a conceptual metaphor. Seldom do experiments in cognitive psychology quantitatively relate the amount of information embedded in stimuli to be processed to mental operations. With the appropriate experimental design, the information-theoretic approach offers a simple and clear measurement of cognitive functions. For example, the executive function can be assessed with tasks that involve uncertainty processing, such as the majority function task (MFT; Fan et al., 2008; Wang et al., 2011; Fan, 2014). In this task, participants are shown a number of left/right arrows and asked to indicate the direction in which the majority of the arrows are pointing. By manipulating the set size and congruency (i.e., the ratio of the number of left/right arrows) in each trial and with assumptions of searching strategies (i.e., exhaustive, self-terminating, or grouping), the per-trial computational load can be quantified with the entropy estimate, "bit." Fan et al. (2008) determined that grouping-search outperformed the other two algorithms in capturing the linear relationship between the reaction time (RT) and computational load in MFT [$RT = a + b \bullet \log_2(s)$, s indicating the average number of arrowheads to be scanned in a trial], where the slope (b) indicates how much more time it takes to process per bit of load increase (i.e., processing efficiency). In contrast, the intercept (a) represents the processing time at the lowest possible load (0 bit), which is the binary choice RT to a single arrowhead.

While MFT demonstrates how information-theoretic approach can metrically quantify efficiency in the executive function, similar applications of metrical quantification have also been documented in the literature of human motor control. The speed-accuracy trade-off has long been considered a fundamental property of human motor behavior (Woodworth, 1899). Fitts (1954) attributed it to the limited capacity of information transmission in the sensorimotor channel. This central limit forces the duration of performing a task proportional to the amount of information (in bits) required for controlling each targeting movement. The amount of information, coined as the index of difficulty (ID), has the form of the ratio of the target distance to its width. Hence the Fitts' law is expressed as follows: movement time (MT) $= a + b \bullet \mathrm{ID} = a + b \bullet \log_2(A/W + 1)$ (direct analogy with Shannon's information theorem; MacKenzie, 1992). The slope (b) indicates how much additional processing time it requires per bit of ID increase. The meaning of the intercept (a) has several interpretations, including unavoidable delay in the psychomotor system (Fitts and Radford, 1966), extra feedback processing time, uncontrollable muscle activity at the beginning or end of the movement task (MacKenzie, 1992), and RT (Fitts and Peterson, 1964). Alternatively, it has also been proposed that Fitts' law is just an approximation of the function of a more general motor circuit model (Beamish et al., 2006), and the intercept in the Fitts' law reflects the consequence of delay processing in the circuit.

The majority function and Fitts' law represent quantitative principles of uncertainty processing in executive and motor functions, respectively. Taking advantages of these two experimental paradigms affording analyses based on the information-theoretic measures can be a way to avoid issues concerning score standardization when carrying out studies comparing performance in different tasks. Regarding the impacts of aging on MFT and Fitts' law, so far no study has documented the difference between the young and elderly groups in MFT performance. However, based on the observations of reduced efficiency in component abilities relevant to MFT, such as visual search, working memory, and conflict resolution, one can reasonably expect elevated intercept and slope of MFT for the elderly group. In contrast, the characteristics of Fitts' law in the later stage of life have been inspected quite thoroughly. It has been quite well-established that, in a variety of Fitts' tasks, the elderly group showed both steeper slopes (Rey-Robert et al., 2012; Temprado et al., 2013) and longer MTs (Welford et al., 1969; York and Biederman, 1990; Teeken et al., 1996; Ketcham et al., 2002). The less efficient processing of the elderly group in both paradigms may or may not have common causes, depending on how well the functional parameters are associated between paradigms, as compared with the young group.

Sleimen-Malkoun et al. (2013) compared Fitts' law and Hick–Hyman's law in young and elderly groups. In contrast to Fitts' law, the Hick–Hyman's law describes the choice reaction time as a linear function of the information entropy of response selection, namely, the binary logarithm of possible Stimulus-Response (S–R) associations, while fixing the complexity of the motor response at the lowest possible level (Hick, 1952; Hyman, 1953; Hawkins et al., 2012). The authors found that while the slopes of the two laws were not statistically separable in the elderly group, the young group showed larger slopes in the Fitts' law than the Hick–Hyman's law. They consider the findings providing evidence for the dedifferentiation view of cognitive aging: owing to the reduction in cognitive resources, distinctive processes at younger age shifted to recruit common resources at an older age, and thus become more similar to each other (Lindenberger and Baltes, 1994; Baltes and Lindenberger, 1997). Although this study demonstrated a novel way of quantitatively contrasting different cognitive domains without transforming the raw data, there are some rooms in the methodological aspects to be further explored, including (1) linear functions were estimated over the group mean RT, which usually overestimates the explained variance; (2) the comparison between tasks qualitatively relied on outcomes

of separate ANOVAs because the levels of the difficulty factor in the two tasks were not identical (i.e., not fully "crossed") and cannot be analytically compared within one single fixed-effect type of model; and (3) trial-by-trial variation within subjects was ignored, which may miss information embedded at the individual level. To deal with these issues, one has to carry out analyses that can take the trial-by-trial variation at the individual level into account and can deal with unbalanced design.

This study will compare aging in the domains of motor and cognitive functions with experimental paradigms manipulating computational load along the same information metric and adopt statistical models that can adequately and quantitatively afford the experimental design. Specifically, the linear functions of the MFT and Fitts' law in the elderly and young groups will be estimated with the generalized linear mixed model (GLMM) to examine the interaction among age, task, and computational load. This will reveal differences between tasks and age groups at the group level (fixed-effect) and at the same time also allows the exploration of correlations between factors, which require estimates of the relationship at the individual level (random-effect). Under the research framework of this study, the dedifferentiation hypothesis would predict not only more significantly different performance–load relationship in the young group than the elderly group but also stronger correlation between individual parameter estimates in the elderly group than the young group.

METHODS

Participants

Thirty-three elderly and 40 young participants were tested in this study after given informed consent. The elderly participants (mean age: 69.9 years, 95% CI = [67.1; 72.6]; mean education: 11.7 years, 95% CI = [10.5; 12.9]) were community dwellers in the close proximity of National Central University (NCU). The young participants (mean age: 22.7 years, 95% CI = [22.0; 23.3]; mean education duration: 16.3 years, 95% CI = [16.1; 16.5]) were undergraduate students of NCU. The young participants were paid 120 NTD (∼4 USD)/h, whereas the elderly participants were given gifts (with a value equivalent to 120 NTD) for participation.

Apparatus and Tasks

Participants were tested in a dim room without being interfered, where they were seated in an adjustable height chair next to a table. They performed an RT task (i.e., the MFT) and a discrete rapid-aiming task (i.e., Fitts' task). Based on the experience from the pilot study, the elderly participants generally feel more difficult to perform the Fitts' task and can take quite a long time to complete it. Therefore, the MFT always precedes the Fitts' task to allow participants to get familiar with the general experimental settings before being challenged with the more difficult task.

The MFT

In this study, in each trial, one, three, or five arrowheads were presented on some of the eight predefined locations on the invisible circular perimeter of a 3° radius. These arrowheads either point uniformly to the right or left or had a direction pointed to by the majority of them (e.g., two left and one right among three arrowheads; one right and four left among five arrowheads). The participant was instructed to determine the "majority direction" and press one of the two mouse buttons to indicate that direction, and the RT was defined as the duration between stimuli onset and the response. Participants practiced for a block of 16 trials before proceeding to the formal experimental trials. Fan et al. (2008) demonstrated that the RT increased as a linear function of the uncertainty that can be quantified by Shannon's entropy and was determined by the composition of the arrowhead directions in a "grouping search" manner. In other words, to solve a trial efficiently, one adopts a strategy to group and sample arrows with a majority size (i.e., more than half of the number of arrowheads in a trial) based on their directions. Accordingly, by defining the majority group size (1, 2, and 3 for set sizes of 1, 3, and 5, respectively) as the information unit and assuming that each sampled group is equivalent to one unit of information, the computational load, namely, the "level of uncertainty," under the grouping search strategy can be quantified as $\log_g(s)$, where g is the majority group size, i.e., a minimal number of arrows pointing in the same direction to be treated as the "majority," and s is the total number of arrowheads to be scanned. To convert this quantity to *bits*, it is multiplied by $\log_2(g)$, i.e., the computation load is $\log_2(g)\bullet\log_g(s)$, which is equivalent to $\log_2(s)$, where s is defined as the average number of arrowheads to be scanned in each condition. By manipulating the number of arrowheads and the composition of their binary pointing direction (3:0, 4:1, and 3:2), we adopted three different levels of computational load [1, 2.91, and 4.91 bits, respectively; see Fan et al. (2008) for how different compositions of pointing directions can be converted into the bit unit]. There were 54 trials for each load, which amounts to 162 trials in total.

The Fitts' Task

In each trial of the Fitts' task, participants moved the mouse pointer on the monitor from the starting location to a target disk as accurately and rapidly as possible. The MT in the Fitts' task was defined as the duration between the time points t_0 and t_1 after the target disk was presented on the screen, in which t_0 indicates the time point when the cursor just moved outside the perimeter of the fixation disk (i.e., 10 pixels from its center), whereas t_1 indicates the time point when the cursor just reached a position of which distance to the center of the target disk shorter than the target radius. The radius and center position of the target disk varied from trial to trial, which resulted in distinct ID, $\mathrm{ID} = \log_2(A/D + 1)$ (Fitts, 1954; later revised by MacKenzie, 1992), where A indicates the distance between the starting location and the center of the target and D indicates the target diameter. The different target disks appeared 5, 10, or 20 pixels in radius, and the distance between starting position and the target center ranged 160, 320, or 480 pixels. The ID here is conceptually equivalent to the load in MFT, and both are quantified in Shannon's entropy, *bit*. To consistently apply the nomenclature of the concept in MFT and Fitts' task, we hereby also call ID as load. Seven different combinations of A and D were adopted in this experiment, which resulted in seven distinct levels of load (2.32, 3.17, 3.7, 4.09, 4.64, 5.04, and 5.61 bits by taking

TABLE 1 | Accuracies and performance time (DV) of Fitts' task and MFT in each combination of age, task, and load.

Age	Task	Load	Accuracy [CI 95%]	DV [CI 95%]*
Old ($N = 33$)	Fitts	2.32	0.96 [0.94, 0.97]	1,268 [1,183, 1,353]
		3.17	0.93 [0.91, 0.94]	1,494 [1,408, 1,579]
		5.04	0.92 [0.90, 0.95]	2,240 [2,039, 2,440]
	MFT	1.00	0.96 [0.94, 0.98]	746 [621, 870]
		2.91	0.98 [0.97, 0.98]	1,365 [1,240, 1,490]
		4.91	0.79 [0.77, 0.82]	1,834 [1,712, 1,956]
You ($N = 38$)	Fitts	2.32	0.89 [0.86, 0.91]	570 [536, 603]
		3.17	0.88 [0.86, 0.91]	706 [673, 739]
		5.04	0.88 [0.85, 0.91]	1081 [1,037, 1,124]
	MFT	1.00	0.97 [0.95, 0.99]	537 [514, 559]
		2.91	0.99 [0.97, 1.00]	1040 [997, 1,083]
		4.91	0.90 [0.87, 0.93]	1476 [1,402, 1,549]

*Values in each cell indicate the mean performance time (*DV indicates movement time in the Fitts' task and reaction time in MFT). Values in the square bracket indicate the upper and lower bounds of the 95% CI for each cell.*

binary logarithm on each level of load). Sixteen replications were presented for each load, except for 3.17 and 4.09 bits that had 32 trials. Participants experienced the balanced number of presentations of different combinations between distance and target size, totaled 144 trials presented in pseudorandom order. Two pairs of different combinations of target distance and diameters happened to render identical IDs, which doubled the number of trials. Before proceeding to the formal experimental trials, participants practiced a block of 16 trials to familiarize themselves with the task. To make the number of load levels comparable between Fitts' task and MFT, only the load levels of 2.32, 3.17, and 5.04 bits were taken into these analyses. It is noted that, in this study, the effective ID [$ID_e = \log_2(A/W_e + 1)$, where $W_e = 4.133 \times SD$; SD indicates the standard deviation of the distribution of the endpoint coordinates] was not computed because, given the definition of MT, the recorded end positions will always be inside the target disk. Therefore, the SD will never exceed the width of the target disk (W). The ID_e in this study, unlike other studies adopting velocity thresholds to determine the end position coordinates, is forced to be equivalent to the designed ID.

Data Analysis

The main dependent variable (DV) in the data analysis is the performance time, namely, RT in MFT and MT in Fitts' task. DV was trimmed adaptively *via* a principled approach that performs trimming in cycles: it first temporarily removes the slowest RT from the distribution and then calculates the mean of the sample. The cutoff value is calculated using a certain number of SD around the mean, with the value for SD being determined by the current sample size. In this procedure, required SD decreases with increased sample size (justification). The temporarily removed RT is then returned to the sample, and the fastest and slowest RTs are then compared with the cutoff and removed if they fall outside. This process is then repeated until no outliers remain, or until the sample size drops below four. The SD used for the cutoff is thus dynamically altered based on the sample size of each cycle of the procedure (Van Selst and Jolicoeur, 1994; Grange, 2015). All trimmed and error trials were excluded. Considering the representativeness of data included in the statistical analysis, participants with any condition that has fewer than 60% of trials remaining in a condition were excluded from further analysis. This resulted in the removal of 13 (9 elderly and 4 young) out of 84 participants from the subsequent analyses. The mean response accuracies of the remaining 71 participants are listed in **Table 1**.

The data were analyzed in the free statistical software environment R (version 3.6.3; R Core Team, 2021) using package *lme4* (version 1.1.23; Bates et al., 2015) for the model fitting procedure. The analysis was carried out with GLMMs on the trial level to optimally accommodate the non-Gaussian nature of the dependent measures and the continuous within-subject predictor (i.e., load) in the experimental design (Hox et al., 2017; Brauer and Curtin, 2018). Fixed-effects included task (i.e., MFT and Fitts), load (2.32, 3.17, and 5.04 bits for Fitts' task; 1, 2.91, and 4.91 bits for MFT), age group (i.e., elderly and young groups), and the interactions among the three factors. Additionally, age (in years), education (in years), and sex (male/female) was treated as nuisance variables where years of age and education were demeaned with respect to age group of each individual to amend the collinearity issue (formula = $DV \sim 1 + age \times task \times load + age_{year} + education_{year} + sex$). We coded task and age effects as +0.5/−0.5 contrasts (i.e., Fitts—MFT, elderly—young) to facilitate interpretations of results and centered load around mean of each participant (i.e., cluster-mean centering; Raudenbush and Bryk, 2002; Brauer and Curtin, 2018) to avoid the confounding between within-subject and between-subject associations (Enders and Tofighi, 2007).

Initially, we specified the random effects of the model in a "maximal manner" (Barr, 2013), where the intercept and all possible slopes are estimated in the random effect structure of the model. The subject was the random factor varying in mean DVs. We also assumed that subjects vary reliably in load and task effects (formula = $\sim 1 + load \times task|subject$). The GLMM assumes that the mean DVs, load effects, and task effects of subjects distributed as inverse Gaussian functions around the

respective fixed effects (i.e., the grand mean DV, the mean slope of load, and the mean difference between RT in MFT and MT in Fitts' task). This specification yields six variance/covariance component parameters for subjects. By comparing the Akaike's Information Criterion (AIC) fit statistics across GLMM with the identity, inverse, and log links, the inverse link turned out to offer the superior performance to the other links (see **Supplementary Table 1**, cf. Lo and Andrews, 2015).

This maximal model was subject to a stepwise procedure of model selection implemented in package *buildmer* (version 1.7.1). We set up the procedure to use the *maximal likelihood* and the *Nelder–Mead* optimizer to find the largest possible GLMM that still converges and then optionally performs stepwise elimination based on the change in log-likelihood as recommended by Matuschek et al. (2017). Summary statistics of the final model were also calculated based on Wald z-scores (**Table 1**).

Both fixed-effect and random-effect estimates of the GLMM are of theoretical interest in this study. As the population-level fixed-effect estimates inform about the effects of the independent variable over the whole group, the relationship among random-effect estimates provides a window for inspecting how different cognitive domains or processing mechanisms interact with each other. It is of theoretical interest to examine the strength of association between the estimates within and between tasks and compare the strength between different age groups: While stronger between-task association suggests more sharing of processing resources across cognitive domains, more vigorous within-task association indicate more interaction between general processing speed and processing efficiency specific to tasks.

RESULTS

The stepwise selection procedure settled on the maximal GLMM with all fixed effects and random effects remained. The total explanatory power of the model, which pitted both the fixed-effect and random-effect variance against the total variance, is substantial (conditional $R^2 = 0.948$). Moreover, the part related to the fixed effects alone (marginal $R^2 = 0.907$) is also very high. Thus, the rest of the "Results" section will describe the fixed- and random-effects and related statistics of the maximal model.

Fixed Effect

All fixed-effect estimates were significant (see **Table 2**). The highest order (three-way) interaction among age, load, and task was significant and is hereby dissected in detail with *post hoc z* tests implemented in the R package *emmeans*. **Figure 1** clearly illustrates that linear functions between DV and load have distinct "slopes" among the combinations of age and task, which is equivalent to say at certain load level(s), performance times differ depending on the particular combination of age and task. Instead of going through each load level, we estimated the slopes of load that serve as linear contrasts at each level of the other two factors. This way, the two-way interaction of age and task on load slopes is effectively the three-way interaction among age, task, and load levels.

With that, in the young group, MFT has a steeper slope (295 ms/bit, 95% CI = [281, 309]) than the Fitts' task (201 ms/bit, 95% CI = [190, 212]; $z = 9.751$, $p < 0.001$); whereas in the elderly group, the two tasks did not differ in slopes (MFT: 326 ms/bit, 95% CI = [309, 342]; Fitts: 352 ms/bit, 95% CI = [327, 377]; $z = -1.680$, $p = 0.334$). As for the comparison of the same task between age groups, both tasks showed significant differences (both $p < 0.001$; see **Table 3**). *Post-hoc* comparisons on the two-way interactions can be found in the **Supplementary Information** and will not be further explained here as they all depend on the significant higher-order interaction explored above.

Correlations Among Random-Effect Estimates

From the random-effect variance–covariance matrix of the GLMM (**Table 2**), it can be observed that the variance of all random-effect estimates (i.e., the three τ_{11} parameters) was quite substantial, suggesting unignorable individual differences in the way task and load modulate performance. Furthermore, there are sizable correlation coefficients between intercept (τ_{00}) and slope of task and the interaction between task and load (τ_{11}). Together these correlations justify closer inspection on how the random-effect parameters associate with one another in different age groups. The GLMM model did not estimate how age modulated the random effects of task and load because it only has two levels and thus not sufficient for fitting as a grouping random-effect. The marginal trend of the load was not computed by the *lme4* package and has to be estimated. Therefore, for each age group, we computed the marginal estimates of slopes and intercepts of the load predictor in both Fitts' task and MFT and then calculated the pairwise correlation coefficients among estimates (**Figure 2**). The elderly group showed a stronger pairwise correlation than the young group in all estimates, and almost all correlation coefficients reached significance (except for between the slopes of both tasks and between the Fitts' intercept and MFT slope). In contrast, for the young group, only the correlation between slopes and intercepts from the same task had a significant correlation. Moreover, the slope–intercept correlation in the Fitts' task is much weaker in the young group than the elderly group ($z = 4.06$, $p < 0.001$). Thus, the pattern of correlation among individual slopes and intercept within and between tasks suggests that elderly and young groups have a distinct relationship between different cognitive domains and processing mechanisms.

DISCUSSION

This study set out to examine how linear functions between processing time and computational load differ between cognitive domains and age groups. The results showed that at the group level, increment in the computational load resulted in statistically identical rates of MT and RT increment in the elderly group. In contrast, the young group showed a greater slope for RT of MFT than for MT of Fitts' law. Moreover, there are stronger associations among individual estimates of

TABLE 2 | Summary table of the final GLMM.

Predictors	Inverse Gaussian/inverse link			
	Estimates	**Standard error**	***CI***	**Statistic**
(Intercept)	−1.090***	0.010	−1.109∼1.071	−110.551
Age	0.475***	0.016	0.442∼0.507	28.855
Age:Load_cm	−0.108***	0.005	−0.119∼−0.098	−19.933
Age:Task	0.370***	0.029	0.313∼0.427	12.657
Age:Task:Load_cm	−0.053***	0.012	−0.076∼−0.029	−4.299
Age_year.dm	0.002	0.001	−0.000∼0.004	1.902
Education.dm	−0.002	0.002	−0.007∼0.003	−0.817
F	Reference			
M	0.015	0.012	−0.008∼0.039	1.267
Load_cm	0.226***	0.003	0.220∼0.231	83.098
Task	−0.141***	0.015	−0.170∼−0.113	−9.678
Task:Load_cm	−0.004	0.006	−0.016∼0.008	−0.599
Random effects				
σ^2	0.01			
$\tau_{00\ subject}$	0.00			
$\tau_{11\ subject.Task}$	0.01			
$\tau_{11\ subject.Load\_cm}$	0.0			0
$\tau_{11\ subject.Task:Load\_cm}$	0.00			
ρ_{01}	0.26			
	−0.95			
	−0.25			
ICC	0.44			
$N_{subject}$	71			
Observations	14,844			
Marginal R^2/conditional R^2	0.907/0.948			
AIC	209348.393			
Log-likelihood	−104652.196			

*The fixed-effect estimates are listed in the upper part of the table, with the factor names indicated by the names in the rows. Age, age groups; Load_cm, load centered at the mean of each individual (weighted by the number of trials in each level); Task, MFT or Fitts' tasks; Age_year.dm, the actual age demeaned with respect to the mean age of each age group; Education_year.dm, the number of years in education demeaned with respect to the mean years of education within each age group; F or M, gender. Row names with factor names separated by colons indicate interaction effects. The middle part of the table lists the random effect parameters. Within-group (residual) variance: σ^2; between-group-variance: τ_{00} (variation between individual intercepts and average intercept); random-slope-variance: τ_{11} (variation between individual slopes and average slope); random-intercept-slope-covariance: τ_{01}; random-intercept-slope-correlation: ρ_{01}. ***$p < 0.001$; ICC, intra-class correlation. See Lüdecke (2021) for detailed explanations of the meaning of each symbol.*

slopes and intercepts for the linear functions in the elderly group than the young group, both within the same task and between tasks.

With unified metrics for quantifying different cognitive functions and principled approaches in statistical analyses, the current findings added to the evidence of dedifferentiation of cognitive aging. It is plausible to assume that slopes of performance time as a function of processing loads indicate the information processing efficiency, and that correlation between slopes in different cognitive domains indicates the extent to how different cognitive domains share processing resources, adopting similar processing strategies, or modulated by some common internal factors (Sleimen-Malkoun et al., 2013). Thus, in the elderly people, the less distinction and the stronger correlation among the functional parameters of processing characteristics in the elderly group suggest a higher extent of commonality in processing resources/strategies between the domains of motor and executive functions.

In terms of potential mechanisms underlying the dedifferentiation, *central slowing* can be one of the candidates. For both MFT and Fitts' task, the elderly group has much steeper slopes and larger intercepts than the young group, indicating that the per-unit increase in processing load requires much more processing time and that even at the lowest possible processing load, the estimate of the processing time of elderly group is still much higher than the young group. It remains to be investigated that whether the longer processing time reflects genuine slowing or a more conservative strategy when facing cognitive challenges. Regardless of the cause, the slowing seems to mask the functional distinctions the cognitive system could have and lead to similar parameters in tasks measuring these functions. In contrast, the young cognitive system is not constrained by factors slowing down various processing streams and thus demonstrates higher efficiencies that preserve the idiosyncratic nature of different cognitive domains, as reflected in the

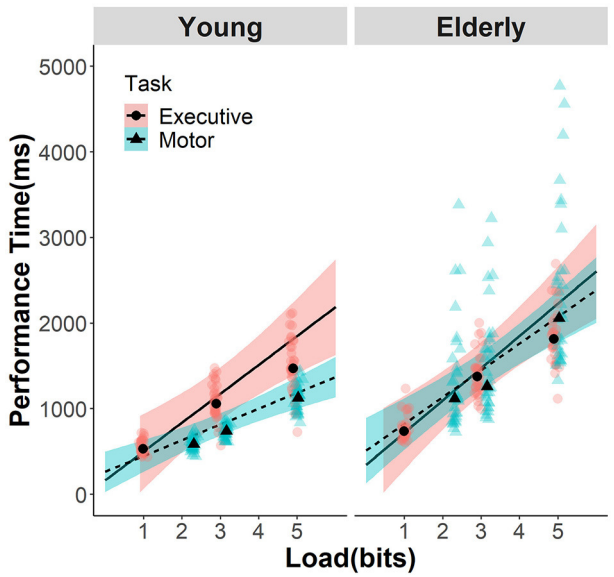

FIGURE 1 | Efficiency functions for movement time in Fitts' task and reaction time in MFT. The data of young participants and GLMM estimates are presented in the left panel, while those for the elderly participants in the right panel. Data and estimates for MFT are indicated with dots, pink color, and solid lines, whereas those for Fitts' task were indicated with triangle, dashed lines, and cyan color. The shaded area indicates the 95% CI of the GLMM estimation. Transparent background symbols indicate the mean data of each individual participants, and the black foreground symbols indicate the mean of each group at the load level included in the analysis.

distinctive slopes between tasks and weaker associations among functional parameters.

Hülür et al. (2015) suggested that the literature on the dedifferentiation hypothesis has reported mixed evidence, which seems to depend on research designs (longitudinal vs. cross-sectional) and nature of the sample (life span cohorts including young and middle-aged participants, or exclusively elderly). While both factors are plausible, in this study, we would like to suggest a third possibility, that is, the way in which different cognitive functions are measured and analyzed can also affect the outcomes and interpretations regarding the differential trends of aging. La Fleur et al. (2018) demonstrated how the inclusion of a test for speed of processing in the correlation with other abilities impacted the tendency of linear dedifferentiation among cognitive domains. As virtually all studies investigating the dedifferentiation hypothesis have relied on distinct ways of quantifying various cognitive domains, inevitably the analyses and interpretations were based on transformed scores. Taking the current results (and also Sleimen-Malkoun et al., 2013) together with previous reports, it is likely that a certain degree of variation in the inconsistencies among reports may be traced back to the distortion of measurements due to transformation and averaging of the performance index.

Although a previous study adopting a similar paradigm (Sleimen-Malkoun et al., 2013) also reached the same conclusion by contrasting the linear function between performance and load in Fitts' law and Hick–Hyman's law, this study extends the comparison across cognitive domains in two important aspects: first, the MFT prompts cognitive processing with an approach different from the multi-alternative S–R conflict resolution in the Hick–Hyman's law, providing convergent evidence to the dedifferentiation hypothesis. Second, in addition to demonstrating the difference between functions, with the aid of GLMM, and this study estimates individual variation in the linear functions and computes the correlations among them. The outcomes of stronger cross-domain and cross-mechanism correlations further support the dedifferentiation hypothesis. This research paradigm allowed more direct cross-domain comparisons than otherwise. Had different cognitive abilities been assessed with tasks that do not share the same scale in computational load, one would have to carry out the comparison based on summarized and transformed results, which may be statistically less powerful and only allows indirect comparison based on certain forms of ranking. This may constrain inferences that can be made with respect to life span trajectories in different cognitive domains.

A few caveats are worthy of note regarding this study: first, although this study aims at contrasting motor and executive functions, the performance in the Fitts' task may additionally involve visuomotor transformation as the participants actually controlled a computer mouse cursor to tap on the targets on the monitor. This experimental setting involved integrating visuospatial, proprioceptive, and motor information from the visual display and the hand holding the computer mouse, which posed a higher challenge than direct tapping. This may explain the much higher intercepts and slopes in this study. Second, the selection of three difficulty levels in processing load may not be optimal for estimating the functional relationship between processing loads and performance time. Although the comparison of the linear functions estimated from all loads higher than 3-bits in the Fitts' task from the current experiment led to similar outcomes and will not change the current conclusion (see **Supplementary Material**), future study may still consider more levels of loads for more robust estimation of the functional relationship. Third, in the Fitts' task, the MT in this study was determined based on geometrical (i.e., the distance to the center of the fixation disk or the target disk) rather than kinematic (e.g., velocity thresholds) landmarks. This makes the estimation of the movement amplitudes and endpoints of movements not entirely based on the ballistic part of the tapping movement and can involve the fine adjustment part in the final "homing-in" phase. Unfortunately, the mouse trajectories were not recorded during the experiment, and thus it is not possible to recover these more purely motor components of the measurements. The recorded endpoints may have underestimated spatial variations as they were confined within the target radius, and the potential inclusion of the homing-in phase may have inflated the MT.

It is a bit ironic to see the core information metric largely missing from experiments in cognitive psychology, a discipline claims to be founded on information processing. However, assessing all kinds of cognitive abilities under information-entropy-based manipulation may require a major overhaul of

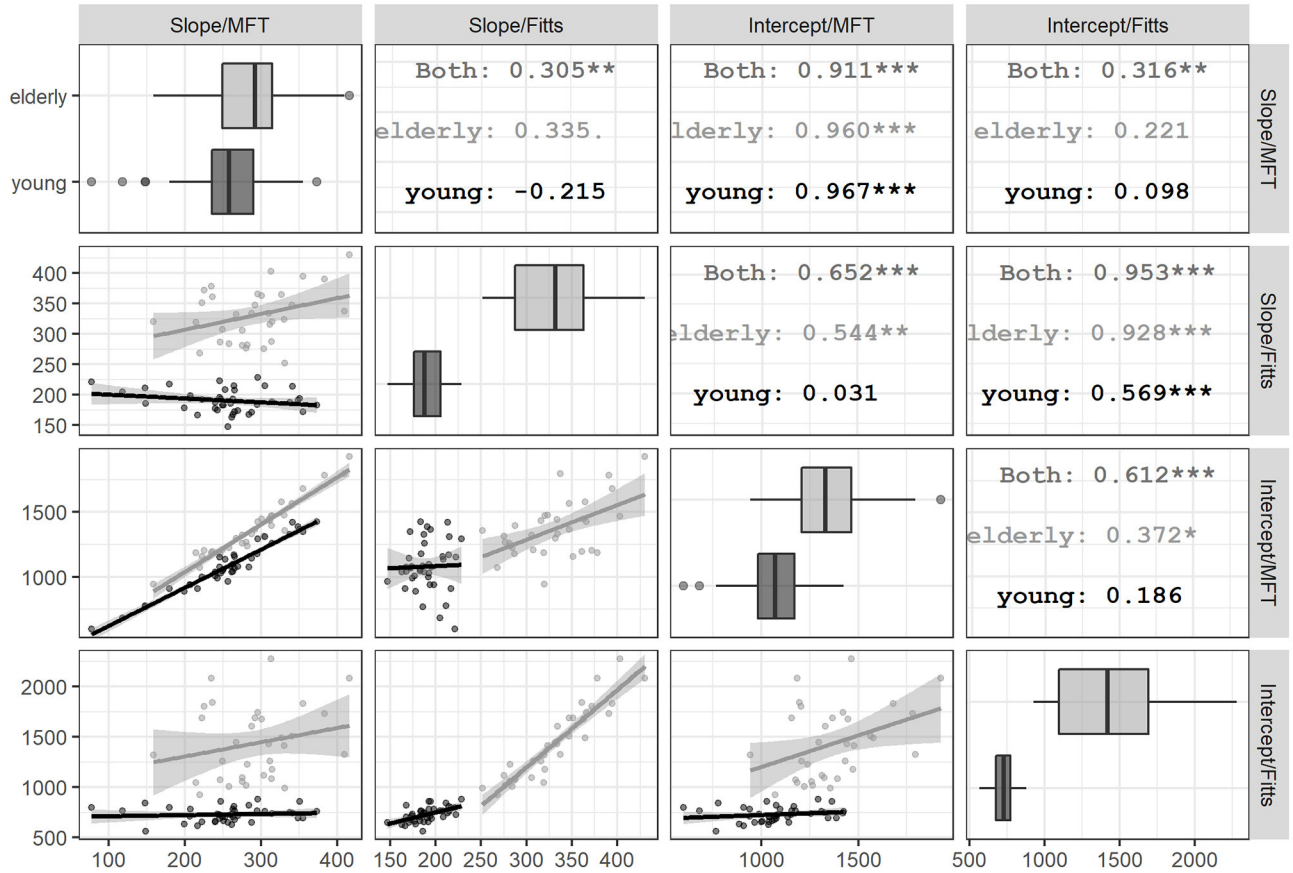

FIGURE 2 | Pairwise correlations among parameters of efficiency functions within each age group. The scatter plots among the slopes and intercepts of MFT and Fitts' task within each age group are displayed in the off-diagonal cells. For the lower half of the matrix, the column and row titles indicate the parameters represented by the *X*- and *Y*-axes of each cell, respectively. For example, the scatter plot at the bottom left corner has column title "Slope/MFT" and row title "Intercept/Fitts," which indicate dots in this cell represent the relationship between these two parameters in each task. In each cell, black line and dark dots indicate the young group, while gray line and light dots indicate the elderly group. The straight lines and shaded area indicate the simple linear regression lines between the *X*- and *Y*-parameters in a cell. Correlation coefficients in the upper-half cells indicate the correlation coefficients of the labeled group in the mirroring cell across the diagonal in the lower-half cells. The asterisks and dots suffixing the coefficients indicate their significance ($p = 0.10$, $^{*}p = 0.05$, $^{**}p = 0.01$, and $^{***}p = 0.001$). As there are 12 coefficients of interest (i.e., six pairwise combinations for the four parameters out of each age group), after correcting for family-wise error, only those with three asterisks should be considered significant. The diagonal cells show the boxplots of the young and elderly groups for the slopes and intercepts of each task.

TABLE 3 | *Post-hoc* comparisons of the fixed-effect three-way interaction.

	Young/MFT	Elderly/MFT	Young/Fitts	Elderly/Fitts
Young/MFT	295			
Elderly/MFT	−31*	326		
Young/Fitts	94***	125***	201	
Elderly/Fitts	−57***	−26	−151***	352

Row and column labels indicate <Age>/<Task>. Diagonal cells: estimates of the load effect (i.e., slope) in the condition indicated by the row and column titles; lower triangle: estimates of differences between the conditions indicated by the row and column labels. Asterisks indicate the significance of the difference (same indication as those in ***Figure 2****). For example, the cell with the row title "elderly/MFT" and column title "young/MFT" has the value of −31, indicating the "column – row" difference in slope between the two age groups for the MFT task [smaller for the column (young) group].*

the tasks originally designed based on operational definitions that have been widely adopted. Fan (2014) proposed several cases of conceptualizing existing tasks for cognitive control under the information-theoretic framework. For example, the conflict effects originated from an interfering dimension, involving word-meaning in the color-word Stroop, flankers in the flanker task, or a global/local feature in a global/local selective attention task, can be treated as uncertainty difference between conflict and

non-conflict conditions. In addition, one can also quantify the uncertainty with the surprise and entropy equations in paradigms investigating the oddball effect, Go/No-Go performance, or task switching, which all involve comparison between responses to high vs. low probability events. It remains a challenge to do likewise in other cognitive domains such as long-term memory and language processing.

CONCLUSION AND APPLICATIONS

This study demonstrates an application of the information-theoretic approach in profiling motor and executive functions. Unlike numerous aging studies that compared different functions or established the relationship between functions *via* contrasting transformed scores or correlating divergent behavioral markers, the findings, in this study, provide supports for the dedifferentiation hypothesis of cognitive aging under the combinations of common efficiency metrics (i.e., bits) and principled statistical framework (i.e., GLMM) and show the group-level interaction among age, task, and computational load as well as the individual-level pattern of association among estimates of the relationship between computational load and performance time. This approach has both theoretical and practical values.

From the theoretical perspective, it may help to resolve inconsistencies in uncovering the changes in the structure of cognitive functions as one ages: as the same processing parameters (i.e., slopes and intercepts) were adopted to assess dedifferentiation of executive and motor domains, it avoids confounding stemming from biases in score transformation and standardization and allows to formulate more accurate assessment on the extent of dedifferentiation (see also Sleimen-Malkoun et al., 2013). This approach may also be applied to other domains that can be quantified with the same scheme. For example, the efficiencies of speech perception and production (e.g., Coupé et al., 2019) or sensory and perceptual processing (e.g., Plumbley and Abdallah, 2006) are both very well-quantifiable under the information-theoretic framework. By designing an appropriate experimental paradigm, the relationship among efficiency functions of various domains can be quantified and contrasted under unified information metrics and statistical models.

With respect to the practical perspective, depicting longitudinal trajectories across cognitive domains in a variety of developmental or clinical populations under this research framework may result in more accurate behavioral markers for identifying coherence between brain activations or rhythms underlying the interaction or dissociation between cognitive functions. The parameters of efficiency functions from different domains can form meaningful features for predicting the progression of cognitive aging or prognosis of neurological diseases.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: OSFHOME: https://osf.io/h57pb/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of National Taiwan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EC is the sole author who conceived and planned the study, coded the experimental tasks, performed the data analysis, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.634089/full#supplementary-material

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