EXERCISE SHAPES UP BRAIN HEALTH

EDITED BY: Frederico C. Pereira, Aderbal S. Aguiar Jr and Henriette van Praag
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EXERCISE SHAPES UP BRAIN HEALTH

Topic Editors:
Frederico C. Pereira, University of Coimbra, Portugal
Aderbal S. Aguiar Jr, Federal University of Santa Catarina, Brazil
Henriette van Praag, Florida Atlantic University, United States

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Table of Contents

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A Multi-Ingredient Nutritional Supplement in Combination With Resistance Exercise and High-Intensity Interval Training Improves Cognitive Function and Increases *N*−3 Index in Healthy Older Men: A Randomized Controlled Trial

Kirsten E. Bell[1], Hanna Fang[2], Tim Snijders[3], David J. Allison[2], Michael A. Zulyniak[4,5], Adrian Chabowski[6], Gianni Parise[2], Stuart M. Phillips[2] and Jennifer J. Heisz[2]*

[1] Department of Kinesiology, University of Waterloo, Waterloo, ON, Canada, [2] Department of Kinesiology, McMaster University, Hamilton, ON, Canada, [3] NUTRIM, Department of Human Biology, Maastricht University Medical Centre, Maastricht, Netherlands, [4] Department of Medicine, McMaster University, Hamilton, ON, Canada, [5] School of Food Science and Nutrition, University of Leeds, Leeds, United Kingdom, [6] Department of Physiology, Medical University of Bialystok, Bialystok, Poland

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Edited by:
Aderbal S. Aguiar Jr., Federal University of Santa Catarina, Brazil

Reviewed by:
Devinder Kaur Ajit Singh, National University of Malaysia, Malaysia
Pawel Zalewski, Nicolaus Copernicus University in Toruń, Poland
Slawomir Kujawski, Nicolaus Copernicus University in Toruń, Poland, in collaboration with reviewer PZ

***Correspondence:**
Kirsten E. Bell
kirsten.bell@uwaterloo.ca

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We aimed to evaluate the effect of multi-ingredient nutritional supplementation, with and without exercise training, on cognitive function in healthy older men. Forty-nine sedentary men [age: 73 ± 6 years (mean ± SD); body mass index: 28.5 ± 3.6 kg/m^2] were randomized to consume a supplement (SUPP n = 25; 1500 mg n−3 polyunsaturated fatty acids, 30 g whey protein, 2.5 g creatine, 500 IU vitamin D, and 400 mg calcium) or control beverage (CON n = 24; 22 g maltodextrin) twice daily for 20 weeks consisting of Phase 1: SUPP/CON followed by Phase 2: 12-week resistance exercise training plus high-intensity interval training, while continuing to consume the study beverages (SUPP/CON + EX). At baseline, 6 weeks, and 19 weeks we assessed cognitive function [Montréal Cognitive Assessment (MOCA)], memory [word recall during the Rey Auditory Verbal Learning Test (RAVLT)], executive functions (working memory inhibition control), and nutrient bioavailability. We did not observe changes to any aspect of cognitive function after Phase 1; however, significant improvements in the following cognitive function outcomes were detected following Phase 2: MOCA scores increased (6 weeks: 23.5 ± 3.3 vs. 19 weeks: 24.4 ± 2.5, p = 0.013); number of words recalled during the RAVLT increased (6 weeks: 6.6 ± 3.6 vs. 19 weeks: 7.6 ± 3.8, p = 0.047); and reaction time improved (6 weeks: 567 ± 49 ms vs. 19 weeks: 551 ± 51 ms, p = 0.002). Although between-group differences in these outcomes were not significant, we observed within-group improvements in composite cognitive function scores over the course of the entire study only in the SUPP group (Δ = 0.58 ± 0.62, p = 0.004) but not in the CON group (Δ = 0.31 ± 0.61, p = 0.06). We observed a progressive increase in n−3 index, and a concomitant decrease in the ratio of arachidonic acid (ARA) to eicosapentaenoic acid (EPA) within erythrocyte plasma membranes, in the SUPP group only. At week 19, n−3 index (r = 0.49, p = 0.02) and the ARA:EPA

ratio (r = −0.44, p = 0.03) were significantly correlated with composite cognitive function scores. Our results show that 12 weeks of RET + HIIT resulted in improved MOCA scores, word recall, and reaction time during an executive functions task; and suggest that a multi-ingredient supplement combined with this exercise training program may improve composite cognitive function scores in older men possibly via supplementation-mediated alterations to $n-3$ PUFA bioavailability.

Clinical Trial Registration: http://www.ClinicalTrials.gov, identifier NCT02281331.

Keywords: resistance exercise training, high-intensity interval training, *n*−3 polyunsaturated fatty acids, protein, creatine, vitamin D, calcium

INTRODUCTION

Dementia is an incurable and debilitating neurodegenerative disorder that severely impairs cognitive function. Currently, dementia affects approximately 50 million people worldwide, however, this number is expected to reach 75 million by the year 2030 (Prince et al., 2015). The impact of this cognitive impairment is widespread and includes reductions in the ability of affected persons to perform activities of daily living, and increases in the financial and psychological burden on families and caregivers (Alzheimer Society of Canada, 2010). Thus, feasible and effective strategies to promote cognitive function and delay the onset of dementia are needed.

Engaging in regular physical activity is associated with a diminished risk of dementia (Barnes and Yaffe, 2011; Ginis et al., 2017). Shorter term (12–24 weeks) exercise training interventions have been shown to improve aspects of cognitive function in older adults, such as executive functions, memory, and processing speed (Smith et al., 2010). Although most research to date has focused on low-to-moderate intensity aerobic exercise training, higher intensity exercise may yield greater cognitive benefits because it stimulates the production of more factors that promote neuroplasticity, such as BDNF (Schmidt-Kassow et al., 2012; Marquez et al., 2015). HIIT improves memory in young adults (Heisz et al., 2017); however, this modality has yet to be tested in older adults. RET has also been shown to improve cognitive function (Liu-Ambrose and Donaldson, 2009). Importantly, improvements in cognitive function may be enhanced when aerobic and resistance exercise are combined (Colcombe and Kramer, 2003).

Nutrition is another modifiable lifestyle factor associated with brain health in aging (Morris, 2012). Observational studies report that populations consuming Mediterranean-style diets, which contain foods rich in polyunsaturated fatty acids (PUFA) including omega-3 ($n-3$) PUFA, have lower dementia rates (Scarmeas et al., 2006) and better cognitive function (Féart et al., 2009) compared to populations consuming typical Western diets. Furthermore, $n-3$ PUFA supplement may independently prevent cognitive decline in older adults (Karr et al., 2011). Higher intakes of vitamin D (with and without calcium) (Balion et al., 2012), protein (van de Rest et al., 2013), and creatine (Pilatus et al., 2009) are also associated with lower rates of cognitive decline and dementia. However, the effects of $n-3$ PUFA, vitamin D, protein, and creatine supplementation on cognitive function are not consistent across studies, and more research is needed to understand the potential interactive effects of these individual components (Calon, 2011). We propose that combining multiple nutritional supplement with an exercise training program that includes both RET and HIIT components may lead to greater improvements in cognitive function compared to each nutrient or intervention alone (Dziedzic, 2006; Franceschi et al., 2007; Peake et al., 2010).

The objective of this study was to examine the effect of a multi-ingredient nutritional supplement containing $n-3$ PUFA, vitamin D (plus calcium), whey protein, and creatine (Bell et al., 2017b), with and without exercise training, on circulating concentrations of BDNF and cognitive function in a group of healthy older men. A secondary objective was to evaluate whether greater nutrient bioavailability was associated with improvements in cognitive function. We hypothesized that, compared to a control drink, our experimental supplement would independently improve cognitive function as well as enhance the typical beneficial effects of exercise training on cognitive function and BDNF concentrations. We further hypothesized that supplementation would increase vitamin D and $n-3$ PUFA bioavailability; and that individuals with higher circulating $n-3$ PUFA at the end of the intervention would also demonstrate greater improvements in cognitive function.

Abbreviations: 1RM, one repetition maximum; ARA, arachidonic acid; BDNF, brain-derived neurotrophic factor; BMI, body mass index; BP, blood pressure; CON, control; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; HDL-c, high-density lipoprotein cholesterol; HIIT, high-intensity interval training; HOMA-IR, homeostatic model of assessment of insulin resistance; IL-6, interleukin-6; LDL-c, low-density lipoprotein cholesterol; MCI, mild cognitive impairment; MOCA, Montréal Cognitive Assessment; $n-3$ PUFA, omega-3 polyunsaturated fatty acid; OGTT, oral glucose tolerance test; RAVLT, Rey Auditory Verbal Learning Test; RET, resistance exercise training; SUPP, supplement; TEE, total energy expenditure; TG, triglycerides; TNF-α, tumor necrosis factor alpha; VO_2peak, peak oxygen uptake.

MATERIALS AND METHODS

Screening and Recruitment

The present study is a secondary analysis from a previously published trial, which examined the effect of multi-nutrient supplementation and exercise training on muscular strength (Bell et al., 2017a,b). Briefly, 49 healthy older men took part in a randomized, double-blind, placebo-controlled parallel group trial

that was conducted between December 2014 and September 2016 at McMaster University. Potential participants were eligible for the study if they: were non-smokers $\geq$ 65 years old; were non-diabetic according to an OGTT, had a BMI in the normal-overweight range, demonstrated normal cardiac function during a maximal exercise stress test; and had not participated in any structured resistance or aerobic exercise training program in the past 6 months. Exclusion criteria included: regular consumption of multi-vitamins, *n*−3 PUFA, whey protein, creatine, calcium, or vitamin D supplements; significant weight loss or gain in the past 6 months; regular use of non-steroidal anti-inflammatory drugs, simvastatin, or anticoagulants; injuries preventing safe participation in an exercise program; diabetes mellitus; cancer; infectious disease; unstable cardiac; and/or gastrointestinal disease.

The primary outcome of the previously published main clinical trial was muscular strength, and as such, sample size was calculated was based on this measure. An increase in leg press isotonic strength of 3.25 kg (standard deviation: 1.5 kg) has been observed during creatine supplementation combined with RET in older adults, versus RET alone (Devries and Phillips, 2014). We assumed a similar response variance in our subjects during a 2-way repeated measures ANOVA; with 80% power and $\alpha = 0.05$, we estimated needing a minimum of 19 subjects per group. To account for a 20% dropout rate, we aimed to recruit 25 subjects per group (50 subjects total).

This study was carried out in accordance with the recommendations of the Canadian Tri-Council policy statement[1] with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Hamilton Integrated Research Ethics Board. This trial was registered at ClinicalTrials.gov (NCT02281331). The CONSORT flow diagram illustrating the movement of subjects through the trial can be found in **Figure 1** (see **Supplementary File S1** for the accompanying CONSORT checklist).

Experimental Design

Participants were randomly assigned to receive either a multi-ingredient nutritional SUPP (n = 25) or a control (CON, n = 24) drink for 20 weeks, as depicted in **Figure 2**. After 6 weeks of consuming their study beverages at home (Phase 1: SUPP/CON), subjects completed a 12-week supervised exercise training program at McMaster University while continuing to consume their assigned beverages (Phase 2: SUPP + EX and CON + EX). We employed a coded (group A versus group B) block randomization scheme (block size: 10 participants) generated using www.randomization.com/ to sequentially allocate subjects to groups in order of enrolment. A key to the randomization code was held by an investigator (SMP) who was not directly involved with subject recruitment, training, or testing. Subjects, as well as investigators who were responsible for recruiting, training, and/or testing subjects, were blind to the individual group assignments. At weeks −1 (baseline), 6, and 19 (post-intervention) we assessed aspects of cognitive function (including verbal memory, executive functions, and processing speed). At each timepoint, we obtained a blood sample the morning after an 8–12 h overnight fast (no food or drink, except for water). Participants refrained from strenuous physical activity for at least 72 h prior to each blood draw. In these blood samples we measured circulating concentrations of BDNF and 25-hydroxyvitamin D [25(OH)D], as well as the phospholipid composition of erythrocyte plasma membranes. Plasma 25(OH)D concentrations and incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into erythrocyte plasma membranes reflect vitamin D and *n*−3 PUFA bioavailability, respectively, and indicate compliance with the supplementation protocol as well as overall nutritional health. Our primary outcomes of interest were circulating BDNF concentrations and cognitive function; the secondary outcomes were plasma 25(OH)D concentrations and erythrocyte phospholipid composition.

Nutritional Supplements

Participants in the SUPP group consumed a multi-ingredient beverage containing: 1,500 mg *n*−3 PUFA (which delivered 700 mg EPA and 445 mg DHA), 30 g whey protein, 500 IU vitamin D, 2.5 g creatine, and 400 mg calcium, twice daily. Participants in the CON group consumed a control beverage containing 22 g of carbohydrate (maltodextrin) twice daily. The exact composition of the SUPP and control drinks has been previously outlined (Bell et al., 2017b). Subjects consumed their first daily beverage within the hour after breakfast, and the second 1 h prior to bed. The control beverages were matched in volume and flavor to the active blend. All study beverages were prepared and labeled in a blinded manner by Infinit Nutrition (Windsor, ON, Canada), and both subjects and researchers were blind to individual group assignments. Participants were instructed not to alter their habitual dietary or physical activity habits (outside of supplementation and exercise sessions included in the protocol) for the duration of the study. To verify adherence to these instructions, participants completed 3-day food records and circulating levels of EPA + DHA and 25(OH)D (see section "Biochemical Analysis") were evaluated at weeks −1, 6, and 19. At each of these timepoints, participants also wore arm-mounted accelerometers (BodyMedia SenseWear Armband, Cardinal Health Canada; Vaughan, ON, Canada) for 72 h to assess habitual physical activity.

Exercise Training

From weeks 7 to 18, subjects engaged in a 12-week progressive exercise training program at the Physical Activity Centre of Excellence (PACE) at McMaster University. Details of the exercise training program have been previously published (Bell et al., 2017b). In brief, subjects completed three supervised exercise sessions per week: whole body RET twice per week (Mondays and Fridays) and HIIT on a cycle ergometer once per week (Wednesdays). At every RET session, participants completed two upper body (chest press and horizontal row on Mondays; lateral pulldown and shoulder press on Fridays) and two lower body exercises (leg press and leg extension on both

[1] http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web.pdf

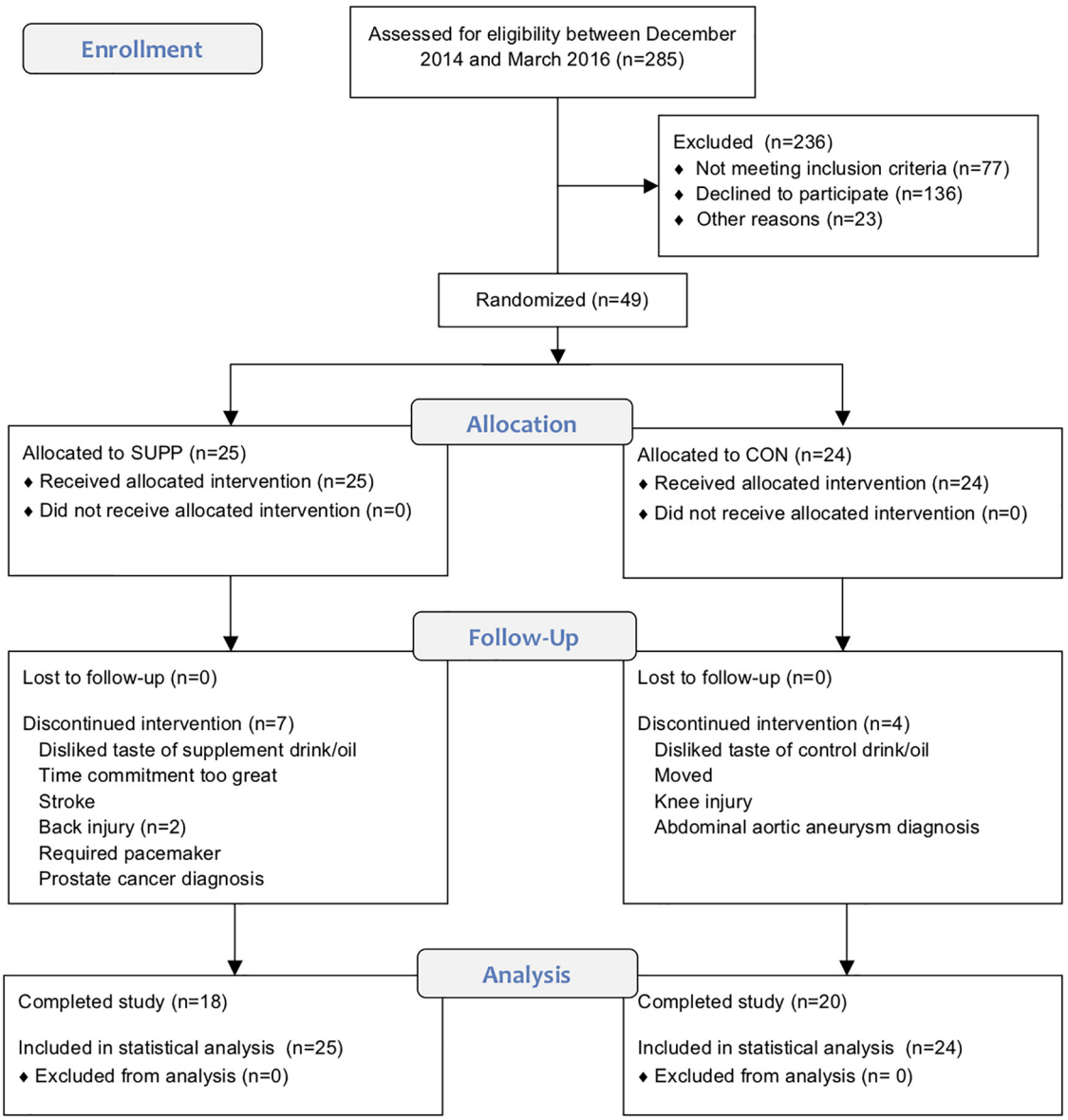

FIGURE 1 | CONSORT flow diagram. This chart depicts the movement of participants through the trial, which we conducted between December 2014 and September 2016.

Mondays and Fridays). Training was performed at 80% 1RM (6–8 repetitions) for three sets, with the last set completed until volitional fatigue. We chose this intensity and repetition range because we have previously shown that heavy loads stimulate greater improvements in muscular strength compared to lighter loads in younger men (Mitchell et al., 2012), and the primary objective of the main clinical trial was to induce strength gains. Participants were allowed approximately 2 min of rest between sets.

During their HIIT sessions, subjects completed 10 × 60 s intervals cycling against a workload predetermined to elicit ∼90% maximal heart rate (HRmax) while maintaining a cadence of ≥90 rpm. HRmax was measured during an incremental exercise test on a cycle ergometer at baseline (week −1), as well as re-assessed immediately prior to the initiation of the RET + HIIT program at week 6. We chose this HIIT protocol over the more traditional Wingate-based (i.e., supramaximal) sprint interval training commonly employed in younger adults, because 10 × 60 s HIIT has previously been shown to be well-tolerated by sensitive populations such as sedentary overweight adults (Gillen et al., 2013), and patients with type 2 diabetes (Gillen et al., 2012) and cardiovascular disease (Currie et al., 2013). HR was measured throughout each HIIT session using a chest strap HR monitor (H7 Heart Rate Sensor; Polar Electro Canada; Lachine, QC, Canada). Intervals were interspersed with 60 s of rest where subjects cycled at a self-selected pace against 25 W.

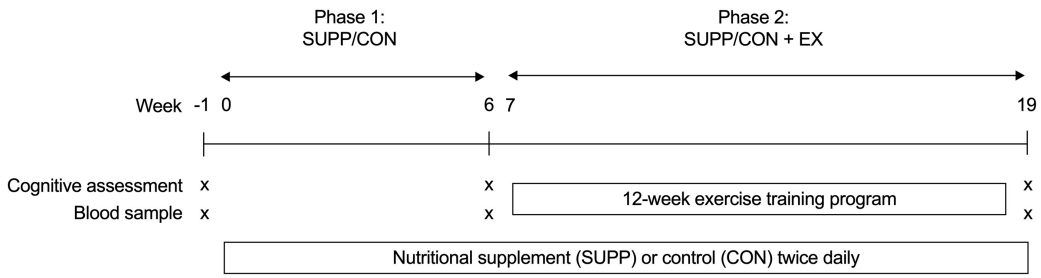

FIGURE 2 | Experimental design. Participants consumed either an experimental supplement (SUPP) or control (CON) beverage twice per day for 20 weeks (weeks 0–19, inclusive). Between weeks 7–18 (inclusive), participants completed a 12-week exercise training program. Exercise training consisted of RET twice weekly (Mondays and Fridays) and HIIT once per week (Wednesdays). At weeks –1 (baseline), 6, and 19 we assessed cognitive function and obtained a blood sample for the measurement of BDNF, 25(OH)D, and erythrocyte plasma membrane phospholipid composition. Phase 1: SUPP/CON took place between weeks 0–6, and Phase 2: SUPP/CON + EX took place between weeks 7–19. SUPP, supplement; CON, control; RET, resistance exercise training; HIIT, high-intensity interval training; BDNF, brain-derived neurotrophic factor; 25(OH)D, 25-hydroxyvitamin D.

All exercise sessions were supervised one-on-one by a member of the research team.

Cognitive Function Assessments

Cognitive function assessments were conducted one-on-one in a quiet room free from interruptions or distractions, using standardized instructions, and primarily administered by one investigator (HF). Each session began with the MOCA, followed by the RAVLT. The RAVLT requires a 30 min delay between Trials 6 and 7 (the immediate and delayed recall tests, which measure short- and long-term verbal memory, respectively), so during this interval participants completed the executive functions and processing speed assessments on a laptop computer[2] (Presentation®). The laptop assessments took 15–20 min to complete, and never caused the interval between the RAVLT Trials 6 and 7 to exceed 30 min. All sessions concluded with the RAVLT delayed recall and recognition tests. In total, cognitive assessment sessions lasted approximately 1 h. To minimize the chance of a learning effect at weeks 6 and 19, three versions each of the MOCA and RAVLT were used in a randomized order, so that each participant completed a new version at each visit.

Montréal Cognitive Assessment

The MOCA (Nasreddine et al., 2005) evaluates a range of cognitive functions including executive functions, memory, language, and orientation, and may be used to identify signs of MCI. Scores $\geq$ 26 (out of 30) are considered normal for healthy adults, and scores $<$ 26 indicate potential cognitive impairment.

Verbal Memory

During the RAVLT (Rey, 1964) participants were instructed to recall as many words as possible, in any order, from a 15-word test list (List A) after listening to a research assistant read the words aloud. This process was repeated 5 times (Trials 1–5), and each trial was scored based on the number of words correctly recalled. Next, subjects listened to a different 15-word distractor list (List B) and were asked to recall as many words as possible. Directly following this, participants were asked to recall as many words as possible from List A without hearing them again (immediate recall, Trial 6). After a 30 min break (during which participants completed the executive functions and processing speed assessments on a laptop, as described below), participants were once again asked to recall as many words as possible from List A without hearing them again (delayed recall, Trial 7). The participants were then presented with a visual recognition list of 30 words (which included the 15 target words from List A) and asked to circle the target words using a pencil. Memory outcomes analyzed were the sum of words recalled during Trials 1–5 (short-term verbal memory), the number of words remembered during the delayed recall test (Trial 7, long-term verbal memory), as well as the number of words recognized from the visual recognition list.

Executive Functions

The Go-NoGo Task assessed working memory inhibition control, a measure of executive functions (Capuana et al., 2012). Participants were presented with 120 uppercase letters in black font appearing one at a time in the center of a white computer screen. They were instructed to press the spacebar ("Go") when they saw any letter *except* four target letters ("NoGo": J, D, V, or M). These target letters comprised one third of the total number of trials. A jittered presentation of a blank white screen preceded the appearance of each letter for 500–1,000 ms, and the length of time each letter appeared on screen was 500 ms. Outcomes analyzed were accuracy (i.e., percentage of correct responses) on the "Go" and "No-Go" trials, as well as reaction time on correct "Go" trials.

Processing Speed

Processing speed was assessed using a Simple Reaction Time Task (Clark et al., 2015) wherein participants were presented with 60 uppercase letters in black font appearing one at a time in the center of a white computer screen. They were instructed to press the spacebar as quickly as possible anytime a letter appeared on the computer screen. As with the Go-NoGo Task, a jittered presentation of a blank white screen preceded the appearance of each letter for 500–1,000 ms, and the length of time each letter

[2]www.neurobs.com

appeared on screen was 500 ms. Accuracy and reaction time were the outcomes analyzed.

Biochemical Analysis

Whole blood was collected into evacuated tubes coated with lithium heparin, and mixed by inversion. Immediately after collection, tubes were centrifuged and the plasma and erythrocyte layers separated. Fasting plasma 25(OH)D concentrations were measured by radioimmunoassay (DiaSorin Canada Inc.; Mississauga, ON, Canada), and fasting plasma BDNF concentrations were measured using a Quantikine Human Free BDNF ELISA kit (R&D Systems, Inc.; Minneapolis, MN, United States).

An increase in the EPA and DHA content of erythrocyte plasma membranes is detectable within 1 week during supplementation with fish oil-derived $n-3$ PUFA (McGlory et al., 2014). Increased membrane content of EPA, in particular, is associated with decreased ARA, a pro-inflammatory eicosanoid precursor (Calder, 2006). Erythrocyte membrane phospholipid composition was measured from the erythrocyte layer as described previously (Dirks et al., 2016). Briefly, total lipids from the samples were extracted (Folch et al., 1957), and thin layer chromatography was used to separate individual classes of phospholipids. Once isolated, phospholipids were methylated with 1 M methanolic sodium methoxide at room temperature for 10 min (Mahadevappa and Holub, 1987), and the FA composition of each class of phospholipids was analyzed by gas chromatography (Hewlett-Packard 5890 Series II System, equipped with a double flame ionization detector, and Agilent CP-Sil 88 capillary column, 100 m, internal diameter of 0.25 mm) (Nawrocki and Gorski, 2004; Bradley et al., 2008). FAs were identified by comparing retention times to those of a known standard, and absolute amounts of individual FAs were calculated with the aid of an internal standard (pentadecanoic acid), which was added to samples before the methylation process. Total amounts of each phospholipid were determined from the sum of FAs in each fraction. EPA + DHA content was determined by summing the total amount of the EPA and DHA in all phospholipid fractions. $n-3$ PUFA abundance was calculated as the ratio of ARA to EPA (ARA:EPA) and as $n-3$ index as follows:

$$n-3\ index\ =\ \left(\frac{EPA+DHA}{total\ FA}\right)\times\ 100$$

Calculations

We computed a composite score that included each cognitive outcome positively impacted by the intervention: MOCA scores, delayed recall (Trial 7, long-term verbal memory) performance on the RAVLT, and reaction time for correct "Go" trials during the Go-NoGo Task. Each of these outcomes was first normalized using their respective baseline means and standard deviations. To match the interpretation of other cognitive outcomes (i.e., with higher values indicating better performance), reaction times were reverse-scored. We then averaged together the normalized values for each outcome to create a composite score of composite cognitive function at weeks −1 (baseline), 6, and 19 (post-intervention).

Statistical Analysis

Statistical analysis was completed using SPSS (IBM SPSS Statistics for Windows, version 23.0; IBM Corp., Armonk, NY, United States). For all measures of cognitive function (MOCA, verbal memory, executive functions, processing speed, and composite cognitive function scores) and all blood analyses [BDNF, plasma 25(OH)D concentrations, and erythrocyte plasma membrane phospholipids] we conducted an intention-to-treat analysis using a linear mixed model with an unstructured covariance matrix, group and time as fixed factors, and subject as a random factor. Age was included as a covariate for all assessments of cognitive function (including composite cognitive function scores) since it is an independent factor related to cognitive performance. In the case of significant group by time interactions, significant between (SUPP or CON) and within (weeks −1, 6, or 19) group differences were identified with Tukey's *post hoc* test. Based on recommendations for human clinical trials with missing data (Elobeid et al., 2009), all participants (completers as well as participants who withdrew prior to week 6 or week 19 testing) were included in the final analyses, and missing values were not replaced. We examined the effect sizes of the changes in measures of cognitive function using Cohen's *D*.

As an exploratory sub-analysis, we used one-sample Student's *t*-tests to evaluate whether the overall change (Δ week 19-baseline) in composite cognitive function scores was significantly different from no change (i.e., zero) in each treatment group (SUPP and CON).

To explore whether consumption of the multi-ingredient experimental supplement was associated with improvements in cognitive function upon completion of the study, we conducted two-tailed Pearson correlations between composite cognitive function scores and the specific nutrients for which bioavailability data was available: $n-3$ PUFA (erythrocyte phospholipid composition) and vitamin D (plasma 25[OH]D_3 concentrations). In an effort to investigate whether one nutrient may exert a greater effect on cognitive function, we then conducted two-tailed partial correlations: (a) between composite cognitive function scores and erythrocyte phospholipids, while controlling for plasma 25(OH)D; and (b) between composite cognitive function scores and plasma 25(OH)D, while controlling for EPA + DHA content. All correlations were performed using data collected at week 19. Although protein, calcium, and creatine were also included in the experimental supplement, we were unable to assess circulating concentrations of these nutrients. As such, protein, calcium, and creatine were excluded from this exploratory correlation analysis.

Data in text and tables are presented as mean ± SD. For all statistical analyses, significance was accepted as $p < 0.05$.

RESULTS

Participants and Compliance

Of the 49 older men randomized, 38 completed the study. Four subjects withdrew prior to week 6 testing (SUPP: $n = 2$; CON: $n = 2$), and seven withdrew partway through the exercise training

program, prior to week 19 testing (SUPP: n = 5; CON: n = 2). Reasons for withdrawal are provided in **Figure 1**. Participants were 73 ± 6 years (mean ± SD) of age and overweight according to BMI (28.5 ± 3.6 kg/m^2). There were no significant differences in baseline physical characteristics between the SUPP and CON groups (**Table 1**). Compliance with the nutrition intervention (assessed via self-report as well as returned sachets) and attendance during the exercise training program were >90% in both the SUPP and CON groups.

Habitual Diet and Physical Activity

Detailed dietary intake data have previously been published (Bell et al., 2017b). Briefly, n−3 PUFA, protein, vitamin D, and calcium consumption all increased significantly by week 6 in the SUPP group, and this increase was maintained throughout the rest of the study. Macro- and micronutrient intake was unchanged throughout the study in the CON group. Energy intake increased (p = 0.004) and TEE decreased (p = 0.006) over time, with no difference between groups. Energy intake increased 10% in both groups at week 6, with no further change following Phase 2. TEE was unchanged in either group at week 6, and decreased 16% following Phase 2.

TABLE 1 | Baseline physical characteristics.

	SUPP (n = 25)	CON (n = 24)
Age (years)	71 ± 5	74 ± 7
Systolic BP (mmHg)	138 ± 19	138 ± 15
Diastolic BP (mmHg)	78 ± 10	78 ± 8
Body mass (kg)	85.3 ± 12.2	84.5 ± 12.1
Height (m)	1.72 ± 0.07	1.72 ± 0.07
BMI (kg/m^2)	28.9 ± 3.9	28.1 ± 3.4
Whole body lean mass (kg)	54.0 ± 5.4	54.5 ± 6.7
Whole body fat mass (kg)	28.2 ± 8.6	26.8 ± 6.8
% body fat	33.6 ± 6.4	32.6 ± 4.8
Leg extension 1RM (kg)	27 ± 7	27 ± 7
Leg press 1RM (kg)	77 ± 17	69 ± 21
VO_2peak (mL/kg/min)	23.8 ± 4.2	24.4 ± 4.6
Peak power (W)	154 ± 25	158 ± 33
Fasting blood glucose (mM)	5.6 ± 0.6	5.8 ± 0.6
2 h blood glucose (mM)	6.8 ± 1.9	7.2 ± 2.1
HOMA-IR	2.1 ± 0.4	2.2 ± 0.5
Total-c (mM)	4.69 ± 1.08	4.83 ± 0.93
LDL-c (mM)	2.74 ± 1.02	2.87 ± 0.86
HDL-c (mM)	1.27 ± 0.31	1.29 ± 0.29
TG (mM)	1.49 ± 0.91	1.50 ± 1.04
Energy intake (kcal/d)	2146 ± 488	2336 ± 553
TEE (kcal/d)	2153 ± 318	2157 ± 627
AEE (kcal/d)	394 ± 258	374 ± 348
Average daily METs	1.3 ± 0.1	1.2 ± 0.3
Depression score[1]	3.2 ± 2.4	3.8 ± 3.6
MOCA score	23.0 ± 2.8	24.2 ± 2.4

[1]*Assessed using the Geriatric Depression Scale (Yesavage et al., 1982). Data are means ± SD. No significant differences between groups. AEE, active energy expenditure; MET; metabolic equivalent; MOCA, Montréal Cognitive Assessment; SUPP, supplement; CON, control; BP, blood pressure; BMI, body mass index; 1RM, one repetition maximum; VO_2peak, peak oxygen uptake; HOMA-IR; homeostatic model assessment of insulin resistance; c, cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; TEE, total energy expenditure.*

Blood Analyses and Nutrient Bioavailability

At baseline, n−3 index was significantly lower in the SUPP versus CON group (p = 0.038; **Table 2**), but both groups fell within the range generally associated with moderate cardiometabolic health (i.e., n−3 index of 4.1–7.9%) (Harris and Von Schacky, 2004; Block et al., 2008). All other erythrocyte-based outcomes (including EPA + DHA content) were similar between groups at baseline. We observed significant group by time interactions for plasma membrane EPA + DHA content, the ratio of ARA:EPA, and n−3 index (all p < 0.001). In the SUPP group, EPA + DHA content and n−3 index significantly increased 60–70% during Phase 1, and a further 10–20% during Phase 2. The ratio of ARA:EPA was significantly reduced following Phase 1, and this reduction was maintained throughout Phase 2. In the CON group, EPA + DHA content, n−3 index, and the ratio of ARA:EPA did not change over the course of the study. At weeks 6 and 19, EPA + DHA content and n−3 index were significantly higher, and the ratio of ARA:EPA was significantly lower, in the SUPP vs. CON group. Importantly, in the SUPP group, n−3 index was ≥8.0% [the range associated with optimal cardiometabolic health (Harris and Von Schacky, 2004; Block et al., 2008)] at both of these timepoints. Total FA content did not change in either group throughout the study.

At baseline, plasma 25(OH)D concentrations were not statistically different between groups (SUPP: 44.3 ± 12.8 nM and CON: 37.6 ± 13.8 nM; **Table 2**). On average, participants' vitamin D status was below adequate, but not deficient [adequate: plasma 25(OH)D > 50 nM; deficient: plasma 25(OH)D < 30 nM (Institute of Medicine, 2011)]. We observed a significant group by time interaction (p < 0.001) such that in the SUPP group plasma 25(OH)D concentrations increased 14% during Phase 1, and a further 13% during Phase 2. As such, mean vitamin D status in the SUPP group was considered adequate at weeks 6 and 19. In the CON group, plasma 25(OH)D concentrations did not change over the course of the study.

Baseline concentrations of plasma BDNF were not different between groups (**Table 2**). We observed a main effect of time (p = 0.007) whereby BDNF did not change during Phase 1, however, concentrations were reduced approximately 45% following Phase 2, with no difference between groups. Despite this, plasma BDNF was not significantly different between weeks −1 and 19 (p = 0.127).

Cognitive Function

Montréal Cognitive Assessment

Prior to beginning the study, mean MOCA scores were below normal cut-points (i.e., <26) in both groups (SUPP: 23.0 ± 2.8 and CON: 24.2 ± 2.4; **Figure 3**), and there were no between group differences at baseline. We observed a main effect of time whereby no changes occurred over the first 6 weeks of the study

TABLE 2 | Blood analyses and nutrient bioavailability.

	SUPP			CON		
	−1 week	**6 weeks**	**19 weeks**	**−1 week**	**6 weeks**	**19 weeks**
Erythrocyte plasma membrane phospholipids						
Total FA (pmol/mg Hb)	10, 551 ± 1959	9792 ± 2048	9640 ± 2006	10, 098 ± 1810	9259 ± 1790	9523 ± 1684
EPA + DHA (pmol/mg Hb)[2]	496 ± 107[a]	798 ± 294[b*]	882 ± 227[c*]	560 ± 128[a]	471 ± 129[a]	458 ± 123[a]
ARA:EPA[2]	140.4 ± 34.0[a]	68.8 ± 33.6[b*]	55.0 ± 47.9[b*]	127.5 ± 40.9[a]	146.9 ± 42.3[a]	150.6 ± 36.7[a]
n−3 index (%)[2]	4.7 ± 0.9[a*]	8.0 ± 1.6[b*]	9.3 ± 2.0[c*]	5.6 ± 1.2[a]	5.1 ± 0.9[a]	4.8 ± 0.9[a]
Plasma 25(OH)D (nM)[2]	44.3 ± 12.8[a]	50.5 ± 14.7[b]	57.1 ± 16.1[c]	37.6 ± 13.8[a]	37.3 ± 12.4[a]	35.6 ± 11.2[a]
BDNF (pg/mL)[1]	391 ± 443[a,b]	384 ± 418[a]	308 ± 240[b]	271 ± 331[a,b]	625 ± 507[a]	273 ± 203[b]

*Data are mean ± SD. Different letters denote significant differences over time within each group. *Significant difference from CON at that time point. [1]Main effect of time ($p = 0.007$). [2]Group-by-time interaction ($p < 0.001$). SUPP, supplement; CON, control; BDNF, brain-derived neurotrophic factor; FA, fatty acid; Hb, hemoglobin; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; n−3, omega-3; 25(OH)D, 25-hydroxyvitamin D.*

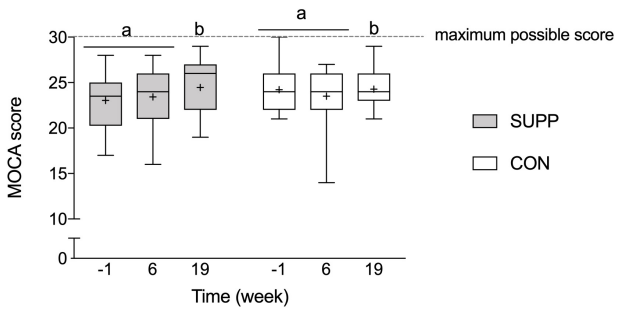

FIGURE 3 | Montréal Cognitive Assessment performance. Scores of 26 or more (out of a possible 30-point total) are considered normal; scores < 26 indicate possible MCI. Boxes (SUPP: gray; CON: white) represent interquartile (25th to 75th percentile) ranges, with the horizontal lines indicating the median. Whiskers represent the maximal and minimal values, and the cross indicates the mean. Dissimilar letters denote changes over time within a given treatment group (SUPP or CON). MOCA, Montréal Cognitive Assessment; MCI, mild cognitive impairment; SUPP, supplement; CON, control.

(Phase 1), however, following the addition of the 12-week exercise training program (Phase 2) we observed a 4% increase in MOCA scores (SUPP: +7% and CON: +0%; $p = 0.01$). Although we report no significant differences between groups at any point in the study, the effect size of the improvement between weeks 6 and 19 was larger in the SUPP vs. CON group (Cohen's D: 0.53 vs. 0.02).

Verbal Memory

At baseline, there were no between group differences in any RAVLT outcomes. We observed a group by time interaction for delayed recognition performance ($p = 0.02$; **Figure 4C**), where the number of words recognized in the SUPP group decreased significantly by 7% at week 6 with no further change following Phase 2. In the CON group, delayed recognition performance was unchanged throughout the study. We observed a main effect of time for long-term verbal memory: the number of words recalled during Trial 7 (delayed recall; $p = 0.047$) did not change over the first 6 weeks of the study (Phase 1), however, following the addition of the 12-week exercise training program (Phase 2) we observed a 15% increase in the mean number of words recalled (SUPP: +19% and CON: +13%; **Figure 4B**), with no difference between groups. We did not observe any change in short-term verbal memory; the sum of words recalled during Trials 1–5 did not change in either group over the course of the study (**Figure 4A**).

Executive Functions

There were no between group differences in any executive functions outcomes at baseline. We observed a main effect of time for reaction time during correct "Go" trials during the Go-NoGo Task ($p = 0.002$; **Figure 5C**) whereby no changes occurred over the first 6 weeks of the study (Phase 1), however, following the addition of the 12-week exercise training program (Phase 2) reaction time decreased approximately 5% (SUPP: −5% and CON: −4%). Although we report no significant between group differences following Phase 2, the effect size of the improvement in reaction time during the Go-NoGo Task was larger in the SUPP vs. CON group (Cohen's *D*: −0.73 vs. −0.44). Accuracy during the "Go" and "NoGo" trials was unchanged throughout the study in either group (**Figures 5A,B**).

Processing Speed

Reaction time (SUPP: 289 ± 51 ms and CON: 300 ± 45 ms) and accuracy (SUPP: 99 ± 1% and CON: 99 ± 2%) during the Simple Reaction Time Task were not different between groups at baseline, and did not change in either group over the course of the study ($p = 0.42$ and $p = 0.43$ for reaction time and accuracy, respectively).

Composite Cognitive Function

Composite cognitive function scores were similar between groups at baseline. We observed a main effect of time ($p < 0.001$), such that no change occurred during Phase 1, however, scores increased by 0.46 (SUPP: +0.54 and CON: +0.40; *data not shown*) over Phase 2, with no difference between groups.

In an exploratory sub-analysis we used one-sample *t*-tests to examine whether the change in composite cognitive function scores over the course of the entire study was significantly different from no change (i.e., zero) in each group. We observed a significant increase in composite cognitive function

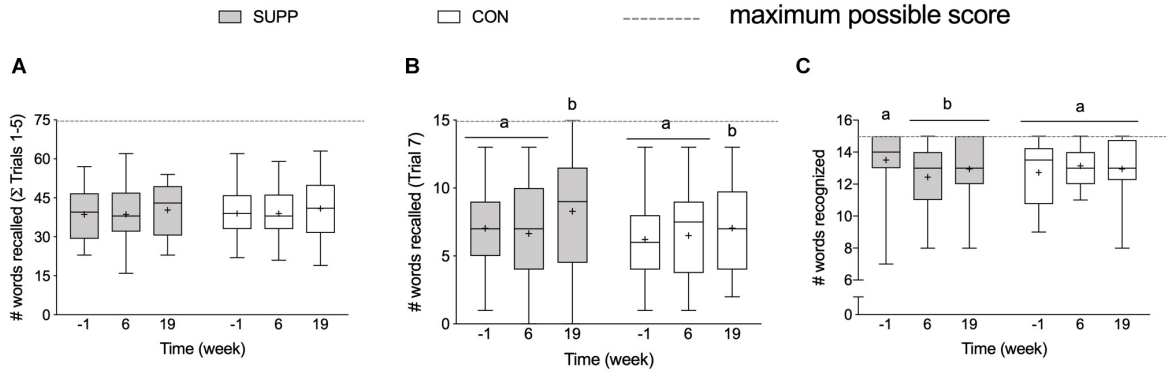

FIGURE 4 | Memory, assessed with the RAVLT. **(A)** Sum of words recalled during Trials 1–5 (out of a possible 75-word total). **(B)** Number of words recalled following a 30 min delay (Trial 7; out of a possible 15-word total). **(C)** Number of words recognized in the 30-word visual recognition list (out of a possible 15-word total). Boxes (SUPP: gray; CON: white) represent interquartile (25th to 75th percentile) ranges, with the horizontal lines indicating the median. Whiskers represent the maximal and minimal values, and the cross indicates the mean. Dissimilar letters denote changes over time within a given treatment group (SUPP or CON). RAVLT, Rey Auditory Verbal Learning Test; SUPP, supplement; CON, control.

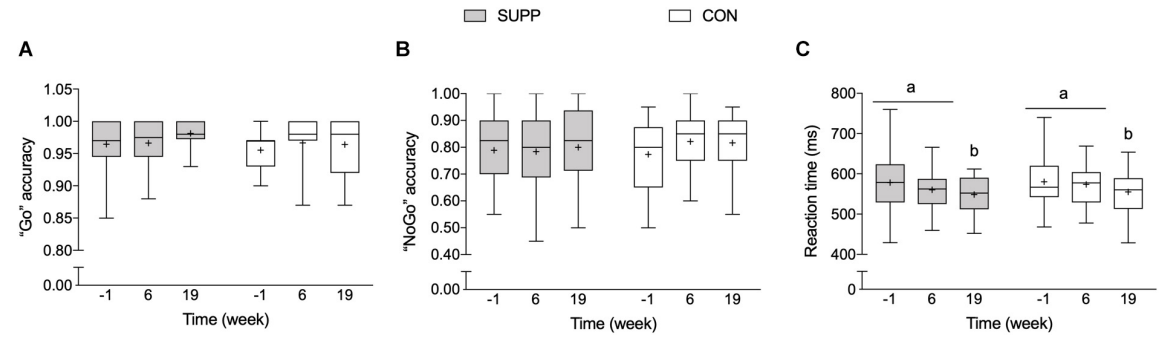

FIGURE 5 | Working memory inhibition control (a measure of executive functions), assessed with the Go-NoGo Task. **(A)** Accuracy (i.e., percentage of correct responses) during "Go" trials. **(B)** Accuracy during "NoGo" trials. **(C)** Reaction time during correct "Go" trials. Boxes (SUPP: gray; CON: white) represent interquartile (25th to 75th percentile) ranges, with the horizontal lines indicating the median. Whiskers represent the maximal and minimal values, and the cross indicates the mean. Dissimilar letters denote changes over time within a given treatment group (SUPP or CON). SUPP, supplement; CON, control.

scores in the SUPP group (Δ week 19-baseline: 0.58 ± 0.62; $p = 0.004$; **Figure 6**); but in the CON group, this increase did not achieve statistical significance (Δ week 19-baseline: 0.31 ± 0.61; $p = 0.06$).

Correlation Analysis

When subjects were collapsed across treatment group, we observed a trend for a positive correlation between end-of-study composite cognitive function scores and erythrocyte plasma membrane EPA + DHA content, as well as between composite cognitive function scores and $n-3$ index. In line with this, composite cognitive function scores and ARA:EPA at week 19 tended to be negatively correlated (**Table 3**). There was no relationship between composite cognitive function and circulating 25(OH)D concentrations even after controlling for EPA + DHA content. However, the correlations between composite cognitive function and erythrocyte phospholipid composition were statistically significant after adjusting for plasma 25(OH)D concentrations.

DISCUSSION

We observed that just 12 weeks of multi-modal exercise training improved cognitive function in a group of previously inactive but healthy older men. Bioavailability of certain components of the experimental supplement ($n-3$ PUFA and vitamin D) increased following 6 weeks of supplementation alone and improved further with the addition of exercise training, yet remained unchanged in the control group. Importantly, upon completion of the exercise training program (at week 19), higher bioavailability of $n-3$ PUFA was associated with greater overall cognitive abilities. Taken together, our results show that 12 weeks of RET + HIIT improved MOCA scores, long-term verbal memory, and executive functions in healthy older men; and suggest that multi-ingredient nutritional supplementation may have contributed to superior improvements in cognitive function following exercise training, possibly via an EPA- or DHA-dependent mechanism.

Consistent with prior research, the specific aspects of cognitive function that benefited from our exercise training intervention

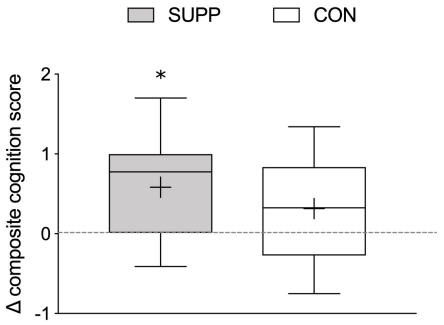

FIGURE 6 | Change in composite cognitive function scores between baseline and 19 weeks. Composite cognitive function scores were calculated using MOCA scores, delayed recall (Trial 7) performance on the RAVLT, and reaction time during correct "Go" trials during the Go-NoGo Task. Boxes (SUPP: gray; CON: white) represent interquartile (25th to 75th percentile) ranges, with the horizontal lines indicating the median. Whiskers represent the maximal and minimal values, and the cross indicates the mean. * indicates a significant difference from no change (i.e., zero). SUPP, supplement; CON, control.

were executive functions and long-term verbal memory (Smith et al., 2010). Reaction time became faster for the more complex executive functions task (the Go-No-Go Task) but not for the Simple Reaction Time Task (Smiley-Oyen et al., 2008). This may reflect a ceiling effect for the Simple Reaction Time Task, which produced consistently high accuracy scores and fast reaction times even at baseline, leaving little room for an intervention-related improvement. We also report a training-related increase in performance on the MOCA, which evaluates a broader range of cognitive functions and may be used as a tool to screen for signs of MCI. At baseline our participants were below normal cutoffs for the MOCA (<26) indicating possible MCI (Nasreddine et al., 2005). Our intervention resulted in average scores that were closer to normal, and in the experimental group the proportion of subjects with MOCA scores < 26 changed from 20/24 (83%) at baseline to 8/17 (47%) at week 19. Conversely, in the control group, this proportion was 16/23 (70%) at baseline and 13/18 (72%) at week 19. Furthermore, the effect sizes of the improvements in the MOCA and executive functions task were larger in the SUPP group. Contrary to our hypothesis, however, we did not detect significant group by time interactions in any of the individual cognitive functions outcomes, most likely due to a lack of statistical power. As such, we performed an exploratory sub-analysis to further probe the relationship between supplementation, exercise, and cognitive function. When the aspects of cognitive function that were positively impacted by exercise training (MOCA scores, long-term verbal memory, and reaction time during the executive functions task) were combined into a composite score, we observed that the change in cognitive performance over the course of the study was significantly greater than zero in the SUPP group, but not the CON group. This suggests that multi-nutrient supplementation may yet be shown to have a beneficial impact on cognitive function (and perhaps an interactive effect with exercise training), however, more studies are needed. We also observed moderate but significant changes to energy intake and expenditure over the course of the study. Total energy intake increased slightly over Phase 1, likely due to supplementation, which added 100–200 kcal per day. The decrease in TEE over exercise training (Phase 2) was most likely due to participants reducing their habitual physical activity outside of the training sessions, possibly due to muscle soreness and/or fatigue. More intriguing, however, are the improvements in cognitive function that occurred over as few as 12 weeks of exercise training.

The relatively rapid change in cognitive function seen in the present study may be related to the potentially additive effects of exercise training combined with our nutritional supplement containing ingredients known to positively impact cognitive function (Scarmeas et al., 2018). After only 6 weeks of supplementation alone we observed increased bioavailability of certain nutrient components of the experimental supplement ($n-3$ PUFA and vitamin D), with no change in the control group. Although these initial changes were not accompanied by improvements in cognitive function, individuals with an erythrocyte plasma membrane phospholipid containing greater EPA and DHA at the end of the study also demonstrated better cognitive performance, after adjusting for circulating vitamin D. These observations suggest, firstly, that $n-3$ PUFA may play a larger role than vitamin D in effecting cognitive improvements in older adults who are not vitamin D deficient. However, since we were unable to measure the bioavailability of the full complement of nutrients in our multi-ingredient supplement, we do not know if $n-3$ PUFA is primarily responsible for any potential nutrition-mediated improvements in cognitive function. Secondly, our findings suggest that cognitive changes may require a longer exposure to nutritional supplementation or the addition of exercise training to become apparent. Improvements in cognitive function were not detected until the end of the study (i.e., after completion of the 12-week RET + HIIT program). As such, we propose that the initial 6 weeks of the study (Phase 1: SUPP/CON) may not have been enough time to alter brain function. Indeed, older adults

TABLE 3 | Pearson and partial correlations between composite cognitive function scores and bioavailable nutrients at week 19 (n = 25).

	EPA + DHA content (pmol/mg Hb)	$n-3$ index (%)	ARA:EPA	Plasma 25(OH)D (nM)	Covariate
Composite	0.35 (0.09)	0.39 (0.06)	−0.37 (0.07)	−0.15 (0.43)[a]	*None*
cognitive function	**0.43 (0.04)**	**0.49 (0.02)**	**−0.44 (0.03)**	–	Plasma 25(OH)D
scores	–	0.18 (0.39)	−0.19 (0.38)	−0.31 (0.14)	EPA + DHA content

[a] *n = 30. Data are r (p). Significant correlations are bolded.*

may need to begin an exercise training program in a comparative state of optimal nutrition (i.e., be "physiologically primed") in order for cognitive function to improve over such a short period of time. This interpretation is consistent with animal models that show reduced neuroplasticity with aging (Calabrese et al., 2013), which would slow the rate of potential improvements in brain function and cognitive function in response to the beneficial effects of nutrition and exercise. Consumption of the supplement alone for a period of more than 6 weeks would test this hypothesis, and is an area for future research.

Our study raises important questions about the potential interactive effects of multi-ingredient nutritional supplementation and exercise training on cognitive function in healthy older men, but additional studies are required. Although we speculate that our observed improvements in cognitive function were driven by the SUPP group, our statistical analyses were underpowered to detect between group differences. *Post hoc* sample size calculations revealed that upwards of 150 subjects per group would have been necessary to identify such an effect. Furthermore, the experimental design of this study allows us to account for and identify certain components of the multi-ingredient supplement that appear most or least influential but our certainty is limited by the design of our supplement. Our correlation analysis was exploratory in nature, and the relationship between $n-3$ PUFA, vitamin D, and cognitive function should be further scrutinized in future studies. This study was conducted on healthy older men who had the physical and cognitive capabilities to complete a challenging RET + HIIT program. This challenging exercise intervention would likely require modification before it could be applied in physically and/or mentally impaired older adults. It is also impossible to discount the potential beneficial effects of social interaction (due to study assessments and exercise training sessions) on cognitive function. Our placebo beverage was an effective nutritional control, but subjects in the CON group received the same amount of social interaction from members of the research team as participants in the SUPP group. Future studies should include a no-interaction control group to account for these potential social effects. Lastly, including women in our study would improve the generalizability of the results. We excluded women from the main clinical trial (the primary outcome of which was muscular strength) to improve the homogeneity of our sample. Some prior work suggests the cognitive benefits from exercise may be stronger in women than men (Colcombe and Kramer, 2003), so the observed effects may be heightened in a study that included women.

CONCLUSION

We report that an exercise training program that combined RET + HIIT improved MOCA scores, long-term verbal memory, and executive functions in a group of older men in as few as 12 weeks. Importantly, the RET + HIIT program was initiated following 6 weeks of prior multi-nutrient supplementation. Our observation of increased $n-3$ PUFA and vitamin D bioavailability in the SUPP group, coupled with a positive correlation between bioavailable $n-3$ PUFA and overall cognitive function, supports the hypothesis that a multi-ingredient nutritional supplement may enhance cognitive adaptations beyond exercise alone. However, this speculation requires support from additional studies. These results may help to inform the development of a dementia invention strategy that uses physical activity with or without nutritional supplementation to improve cognitive functions for the growing number of older persons.

DATA AVAILABILITY

All relevant data are within the paper and its **Supplementary Material Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Canadian Tri-Council Policy statement (http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web pdf) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Hamilton Integrated Research Ethics Board.

AUTHOR CONTRIBUTIONS

KB, TS, GP, SP, and JH conceived of and designed the study. KB, HF, TS, and MZ collected the data. KB, DJA, AC, and JH analyzed the data. KB and JH wrote the manuscript. 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.2019.00107/full#supplementary-material

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Effects of Maternal Voluntary Wheel Running During Pregnancy on Adult Hippocampal Neurogenesis, Temporal Order Memory, and Depression-Like Behavior in Adult Female and Male Offspring

Suk-Yu Yau[1,2], Thomas Ho-Yin Lee[1], Douglas Affonso Formolo[1], Wing-Lun Lee[1], Leo Chun-Kit Li[1], Parco M. Siu[3] and Chetwyn C. H. Chan[2,4]*

[1] Department of Rehabilitation Sciences, The Hong Kong Polytechnic University, Hong Kong, Hong Kong, [2] University Research Facility in Behavioral and Systems Neuroscience, The Hong Kong Polytechnic University, Kowloon, Hong Kong, [3] Divison of Kinesiology, School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong, [4] Applied Cognitive Neuroscience Laboratory, The Hong Kong Polytechnic University, Kowloon, Hong Kong

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***Correspondence:**
Suk-Yu Yau
sonata.yau@polyu.edu.hk; sonatayau@gmail.com

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Research suggests that maternal exercise in pregnancy may have beneficial effects on the brain function of offspring. This study sought to determine if voluntary wheel running during pregnancy improves depression-like behavior, temporal order memory, and hippocampal neurogenesis in both female and male offspring mice. Pregnant mice were allowed to run voluntarily by introducing running wheels into the housing cages throughout the gestational period. Male and female mice offspring at the age of 8- to 9-week-old were then tested on the temporal order task and forced swim test, then euthanized for immunostaining for examining adult hippocampal cell proliferation and neuronal differentiation. Results showed that both male and female pups had reduced depression-like behavior, while only male offspring demonstrated improvement in temporal order memory. Immunostaining revealed that male offspring showed an increase in the number of immature neurons in the ventral hippocampus, whereas female offspring showed enhanced cell proliferation in the dorsal hippocampus. These findings indicate that maternal voluntary wheel running benefits both female and male offspring on reducing depression-like behavior, but with gender effect on promoting hippocampal cell proliferation, neuronal differentiation, and temporal order memory.

Keywords: hippocampus, adult neurogenesis, maternal exercise, depression-like behavior, gender, offspring

INTRODUCTION

Maternal physical exercise during pregnancy lowers the risk of cancer, cardiovascular diseases, and metabolic disorders of the offspring (Blaize et al., 2015). Additionally, maternal exercise can elicit long-lasting and positive effects on the offspring brain during the critical period of fetal brain development (Robinson and Bucci, 2012). In humans, maternal exercise not only improves the growth of fetus and placenta, but also promotes brain development, connectivity, and enhances

cognitive functions in offspring in their later life. For example, maternal exercise during pregnancy has been shown to improve intelligence and the language skills of children when they are 5 years old (ClappIII, 1996). Also, maternal physical exercise training including jogging, yoga, weight-lifting, and aerobics during pregnancy promotes language skills in the offspring as assessed when they are 15 months old (Jukic et al., 2013). The results have suggested a long-lasting improvement and transgenerational neuroplasticity induced by maternal exercise in human brains.

The hippocampus is a brain region involved in memory formation and emotion regulation. It is anatomically divided into the dorsal and ventral hippocampi, which are associated with spatial navigation and affective-related functions, respectively (Moser and Moser, 1998). Physical exercise is renowned for improving learning and memory, and reducing depressive behaviors (van Praag et al., 1999a; Sahay and Hen, 2007). These improvements are partly linked to enhanced adult neurogenesis in the hippocampal dentate gyrus (DG) (van Praag et al., 1999b; Kronenberg et al., 2003; Eadie et al., 2005; Erickson et al., 2011). Animal studies have demonstrated that maternal physical exercise enhances cognitive performance and hippocampal neurogenesis in the adult male offspring (Bick-Sander et al., 2006; Lee et al., 2006; Kim et al., 2007; Akhavan et al., 2013; Robinson and Bucci, 2014; Torabi et al., 2017). Moreover, 10 min of forced swimming daily (Lee et al., 2006), 30 min of treadmill running once per day (Kim et al., 2007), or voluntary wheel running (Akhavan et al., 2013; Robinson and Bucci, 2014) thorough the pregnancy period was shown to positively influence the offspring. On the other hand, maternal stress during pregnancy leads to sex-specific detriments in the offspring behavioral performance (Koenig et al., 2005; Schulz et al., 2011; Luine et al., 2017), which is shown to be sex-specific. Similarly, the beneficial effects of maternal physical exercise could also be sex-specific (Titterness et al., 2011). Such sex differences in response to maternal exercise in offspring, however, have not yet been examined.

Taken previous studies together, we hypothesized that maternal voluntary wheel running during pregnancy could improve hippocampal-dependent cognitive performance and hippocampal neurogenesis in a sex-specific manner in the adult offspring. In the present study, we sought to extend the research in this area by Blaize et al. (2015) evaluating the effects of physical exercise during pregnancy on cognitive abilities and depressive behavior in the adult offspring; (Robinson and Bucci, 2012) examining whether behavioral improvements are linked to enhancement in adult hippocampal neurogenesis in the dorsal and ventral DG, and (ClappIII, 1996) determining if there are any sex differences in these effects.

MATERIALS AND METHODS

Animals and Experimental Design

All experimental procedures were approved and followed the guidelines of the Animal Subjects Ethics Sub-Committee from The Hong Kong Polytechnic University. C57BL/6J mice had standard chow and water *ad libitum* in the animal holding room in a 12-h light-dark cycle (lights on at 9 a.m.). Animals were group-housed, as previously described (Yau et al., 2014), in order to avoid the stress induced by social isolation that can affect adult neurogenesis.

Following a 2-day acclimation period, two females were placed with one male. Pregnancy was confirmed by the presence of seminal plugs. After confirmation, three running wheels were introduced in each cage until the pups were born. Pups were weaned, separated from their parents, and placed in different cages according to their sexes on the postnatal day 28 (PND), without running wheels. Male and female offspring at the age of 8- to 9-week old were then subjected to the temporal order task and the forced swim test in two consecutive days, respectively, and were euthanized the day after (**Figure 1**).

Behavioral Tests

The adult offspring mice were pre-handled for 7 days to reduce the handling stress as previously performed (Yau et al., 2016). On the next day, they were acclimatized to the behavioral test room for 2 h and then to the empty temporal order testing apparatus for 15 min (Width × Length × Height: 40 cm × 40 cm × 30 cm). Animals were subjected to the temporal order task on the next day, followed by the forced swim test on the day after (only one test was performed per day).

Temporal Order Task

This test assesses temporal order memory. Mice received three training sessions to explore three copies of a new set of objects (sets 1, 2, 3, respectively) for 5 min, followed by a 5-min

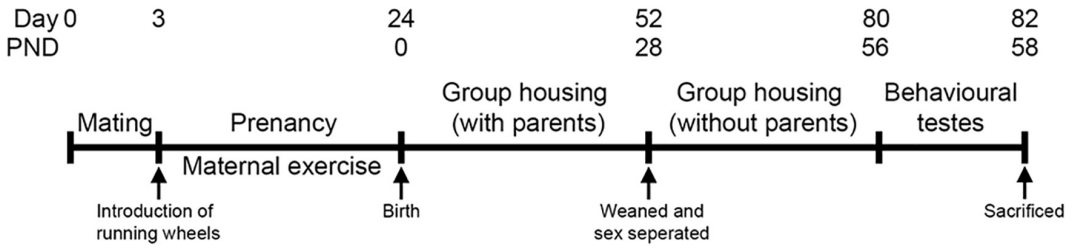

FIGURE 1 | Experimental timeline. Two females were housed with one male in a holding cage. Pregnancy was confirmed by the presence of seminal plugs. After confirming pregnancy, three running wheels were placed in the cages until the pups were born. Pups were weaned and separated on the postnatal day 28. Both male and female offspring were used for the experiments when they aged 8- to 9-week old, and then euthanized at the experimental end point.

intermission interval in transfer cage. During the 5-min test session, mice were allowed to explore a copy of the object set 1 and object set 3. More time spent exploring the first object presented (object set 1 as relatively more novel object) relative to the most recent explored object (object set 3 as recently familiar object) indicates normal temporal order memory. Exploration ratio of the objects was calculated as (exploration time of object 1 or 3)/(exploration time of object 1 + 3). Exploration index was calculated as (exploration time of object 1 – 3)/(exploration time of object 1 + 3) (Yau et al., 2016).

Forced Swim Test

A mouse was transferred to a cylinder (height: 30 cm; diameter: 15 cm) filled with water at room temperature (24–25°C) and videotaped for 6 min as previously performed (Yau et al., 2014). The time spent immobile during the last 4 min of the testing session was scored by an observer blind to the treatment conditions and presented as an indicator for depression-like behavior. Immobility was defined as any movement beyond what was needed to keep the head above the water (Yau et al., 2014, 2018).

Tissue Preparation

Mice were deeply anesthetized with isoflurane (1–3% vaporizer, Abbott Laboratories). Upon collection of trunk blood, they were perfused with 0.9% saline and 4% paraformaldehyde (PFA) in 0.01 M phosphate buffered saline (PBS). The brains were post- fixed in 4% PFA overnight at 4°C and were then transferred to 30% sucrose until they sank. The brain sections (1-in-6 series, 30-μm thickness) were obtained coronally using a vibratome (Leica VT1200S). The sections were stored in the cryoprotectant at 4°C until use.

Immunohistochemical Staining

The free-floating brain sections were retrieved in citrate buffer (pH 6.0) at 95°C for 10 min. After washing in 0.01 M PBS, the sections were incubated overnight with rabbit polyclonal anti-Ki-67 (1:1000; Abcam) antibody at room temperature, and then incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, United States) in blocking solution containing 5% normal goat serum for 2 h at room temperature. After three washes in PBS, brain slides were incubated with an avidin-biotin complex (Vector Laboratories) for 2 h as previously described (Gil-Mohapel et al., 2013). The Ki-67 staining was visualized by peroxidase method (ABC system, Vector Laboratories, Burlingame, CA, United States) and diaminobenzidine kits (DAB kits, Sigma-Aldrich, United States) as previously described (Gil-Mohapel et al., 2013). For doublecortin (DCX) staining, sections were incubated with anti-DCX (1:200; Chemicon) antibody at

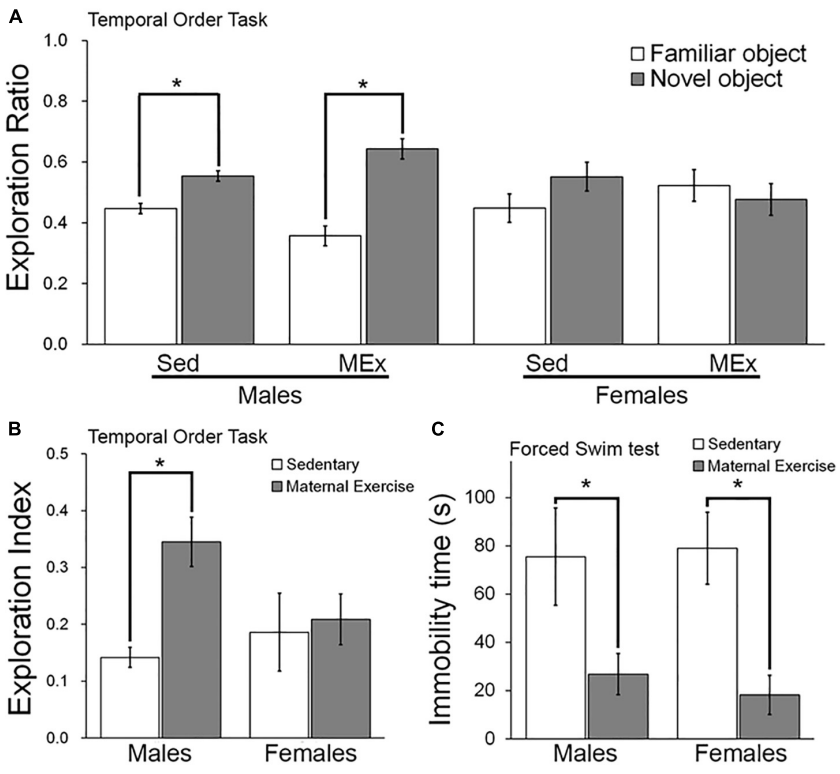

FIGURE 2 | Behavioral performance of offspring in Sedentary (Sed) and Maternal Exercise (MEx) groups. **(A)** Male and female offspring from both Sed and MEx group showed a higher exploration ratio to the novel object than to the familiar object in the temporal order task. **(B)** Male offspring from MEx group displayed a significant enhancement in temporal order memory when compared to Sed group. This effect was absent in female offspring. **(C)** Both male and female MEx pups showed a decrease in depression-like behavior in the forced swim test when compared to Sed pups $^{*}p < 0.05$; $n = 5$–7 per group.

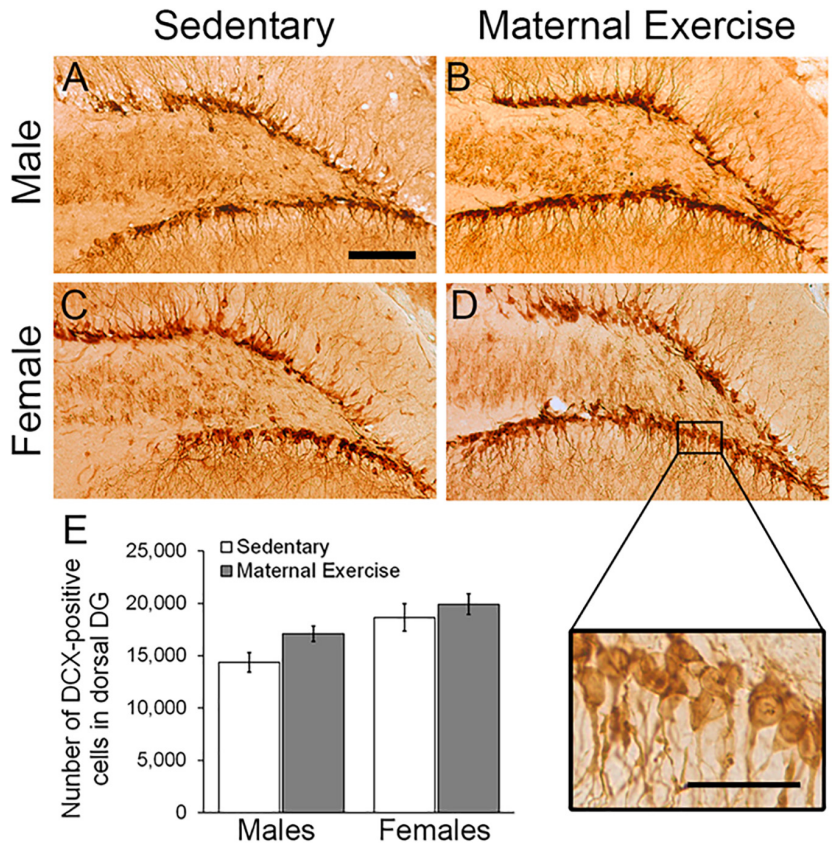

FIGURE 3 | Effect of maternal exercise on number of immature neurons in the dorsal DG of offspring. Representative images of DCX positive cells in the dorsal DG **(A,C)** sedentary control and **(B,D)** maternal exercise group at 20 × (scale bar = 100 μm) and 40 × (scale bar = 50 μm) magnification. **(E)** No significant effect of maternal exercise on the number of immature neurons in the offspring was observed when compared to sedentary control in both sexes. *n* = 5–7 per group.

room temperature overnight. After washing three times in PBS, the brain slices were incubated with biotinylated rabbit anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA, United States) in blocking solution containing 5% normal rabbit serum at room temperature for 2 h, followed by three washes in PBS and 2-h incubation with avidin-biotin complex at room temperature. Positive staining was then visualized using the same DAB kit as for the Ki-67 staining.

Quantification of Ki67$^+$ and DCX$^+$ Cells

All quantification analyses were performed by a trained researcher using a Nikon series Eclipse H600L microscope. Total number of labeled cells located in the hippocampal DG was counted in a sample blinded manner as previously performed (Gil-Mohapel et al., 2013). Briefly, all DAB-positive cells present within two cell diameters of the subgranular zone (SGZ) were counted. Total number of DCX- or Ki-67-immunopositive cells present in the SGZ of either the dorsal DG (from Bregma −1.34 to −2.54), or the ventral DG (Bregma −2.54 to −3.40) sub-regions were quantified as previously performed (Gil-Mohapel et al., 2013). Total number of positive cells were estimated by multiplying the average number of labeled cells per DG section by the estimated number of 30-μm-thick sections containing either the dorsal or the ventral DG, as previously performed (Gil-Mohapel et al., 2013).

Statistical Analysis

Independent Student *t*-test was performed to compare between mean values of two experimental groups. To evaluate the effects of maternal voluntary wheel running and sex, a two-way ANOVA followed by Fisher's *post hoc* test was used. The statistical analyses were performed using the SPSS statistic 25.0 software (SPSS Inc., Chicago, IL). A probability (*P*) value lower than 0.05 was considered statistically significant. Data were shown as means ± SEM.

RESULTS

Maternal Voluntary Wheel Running Improved Temporal Order Memory in Male Offspring, but Not Female Offspring

We first examined the temporal order memory of the offspring. Both male (**Figure 2A**; t_6 = 9.999 and t_{10} = 10.33 respectively) and female offspring (**Figure 2A**; t_8 = 3.451 and t_4 = 3.809

respectively) showed a significantly greater preference toward the novel object, represented by a longer exploration ratio of the Object 1. In addition, male offspring from the maternal wheel running group had a significant increase in the exploration index, suggesting a better temporal order memory as compared to the sedentary group (**Figure 2B**; $p = 0.026$). However, the maternal wheel running did not enhance temporal order memory in the female offspring (**Figure 2B**; $p = 0.810$).

Maternal Voluntary Wheel Running Reduced Depression-Like Behavior in Both Male and Female Offspring

Two-way ANOVA analysis indicated a significant main effect of maternal exercise on reducing depression-like behaviour in offspring [**Figure 2C**; $F(1,20) = 14.45$, $p = 0.001$], but no significant main effect of sex [**Figure 2C**; $F(1,20) = 0.031$, $p = 0.862$]. *Post hoc* analysis demonstrated that maternal exercise significantly reduced depression-like behavior in both male (**Figure 2C**; $p < 0.05$) and female offspring (**Figure 2C**; $p < 0.05$). However, there was no interaction between offspring sex and maternal exercise treatment [**Figure 2C**; $F(1,20) = 0.179$, $p = 0.677$].

Maternal Voluntary Wheel Running Increased the Number of Immature Neurons in Ventral DG, but Not Dorsal DG of Male Offspring

Two-way ANOVA analysis revealed that maternal exercise did not increase the number of immature neurons in the dorsal DG in both male and female offspring [**Figure 3**; main effect of exercise: $F(3,23) = 3.982$, $p = 0.060$, main effect of sex: $F(3,23) = 12.594$, $p = 0.002$]. However, there was a significant main effect of sex on increasing the number of immature neurons in the ventral DG [**Figures 4A–E**; $F(3,23) = 18.017$, $p = 0.0004$]. *Post hoc* test revealed that maternal exercise significantly increased the number of DCX positive cells in the ventral DG of the male offspring when compared to the sedentary group (**Figure 4E**; $p < 0.05$), but not in the females ($p = 0.810$).

Maternal Voluntary Wheel Running Increased Hippocampal Cell Proliferation in Dorsal, but Not Ventral DG of Female Offspring

Maternal exercise increased the number of the dorsal DG proliferating cells in sex-specific manner, as represented by a

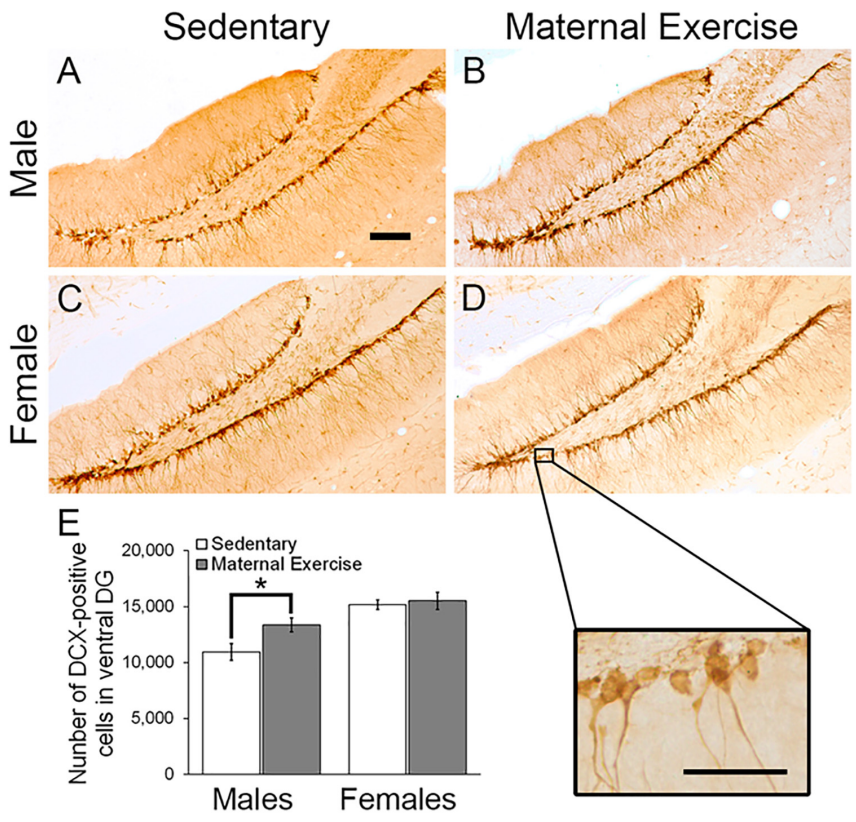

FIGURE 4 | Effect of maternal exercise on number of immature neurons in the ventral DG of offspring. Representative images of DCX positive cells in the ventral DG **(A,C)** sedentary control and **(B,D)** maternal exercise group at 20 × (scale bar = 100 μm) and 40 × (scale bar = 50 μm) magnification. **(E)** Maternal exercise significantly increased the number of immature neurons in male offspring when compared to male sedentary control, while this effect was not observed in females. *$P < 0.05$; $n = 5$–7 per group.

main effect of sex in the two-way ANOVA [**Figures 5A–E**; main effect of exercise: $F(3,22) = 2.036$, $p = 0.170$, main effect of sex: $F(3,22) = 10.470$, $p = 0.004$]. *Post hoc* analysis showed that maternal exercise increased the dorsal DG cell proliferation levels in the female offspring (**Figure 5E**; $p < 0.05$), but not in the males (**Figure 5E**; $p = 0.950$). Maternal exercise did not show significant effect on the ventral DG [**Figure 6**; $F(3,23) = 2.657$, $p = 0.076$], either for male ($p = 0.961$) or female offspring ($p = 0.808$) when compared to control.

DISCUSSION

Our results demonstrated that maternal exercise during pregnancy, in terms of voluntary wheel running, significantly reduced depression-like behavior in both adult male and female offspring. Interestingly, temporal order memory improved only in the male offspring in concurrent with a significant increase in the number of immature neurons in the ventral DG. In contrast, maternal voluntary wheel running increased the number of proliferating cells in the dorsal DG of the female offspring, without affecting the temporal order memory performance.

Previous rodent studies have documented the benefits of voluntary wheel running on cognitive function and hippocampal neurogenesis (van Praag et al., 1999b; Kronenberg et al., 2003; Eadie et al., 2005; Erickson et al., 2011). An earlier study revealed that the beneficial effect of physical activity could be passed from the mother to their progeny (Bick-Sander et al., 2006). Our investigation extends these findings by showing that maternal physical activity improved hippocampal cell proliferation and neuronal differentiation as well as cognitive functions in the adult offspring. The current data is also consistent with previous reports showing the positive impacts of exercise on depression-like behavior, learning and memory that persisted into adulthood (Bick-Sander et al., 2006; Lee et al., 2006; Kim et al., 2007; Dayi et al., 2012; Akhavan et al., 2013; Torabi et al., 2017). However, Dayi et al.'s (2012) study showed improved spatial learning memory, as indicated by the Morris water maze test, in both male and female adult offspring. Contradicting that, in our study only the male offspring showed improvement in the hippocampal-dependent memory, as reflected by a better performance in the temporal order task. Such contradictions could be explained by differences in the tasks used in each study, as well as in the specific maternal exercise protocols used.

In spite of the multiplicity of exercise training paradigms, including forced treadmill running (Bick-Sander et al., 2006; Kim et al., 2007), forced swimming (Lee et al., 2006), and voluntary wheel running (Akhavan et al., 2013; Robinson and Bucci, 2014),

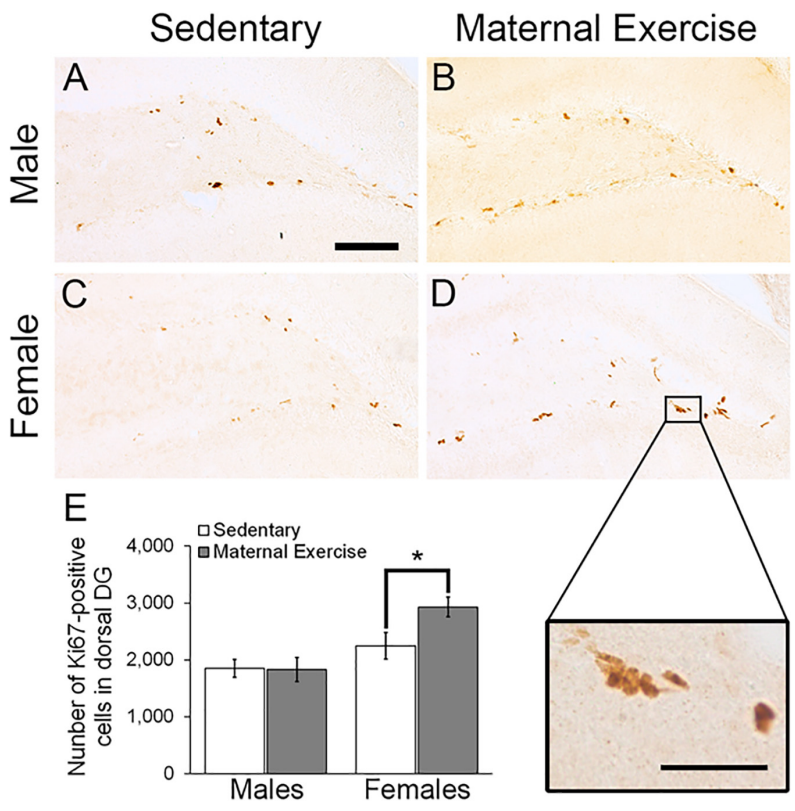

FIGURE 5 | Effect of maternal exercise on cell proliferation in the dorsal DG of offspring. Representative images of Ki-67 positive cells in the dorsal DG from **(A,C)** sedentary control and **(B,D)** maternal exercise group at 20 × (scale bar = 100 μm) and 40 × (scale bar = 50 μm) magnification. **(E)** Maternal exercise significantly increases cell proliferation in dorsal DG of female offspring when compared to female sedentary control, while this effect was not observed in male offspring. *$P < 0.05$; $n = 5$–7 per group.

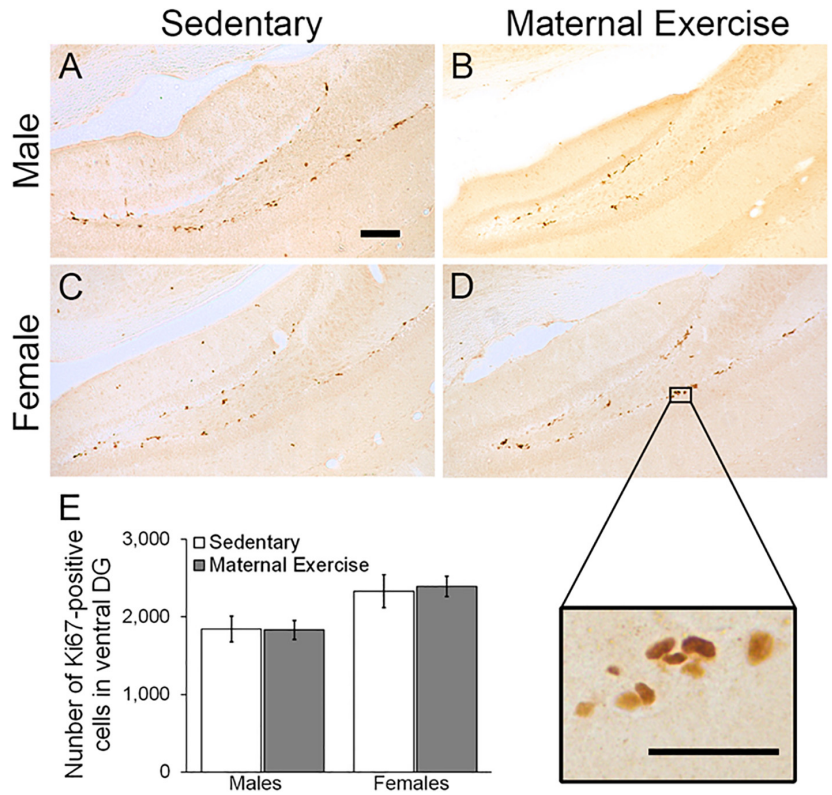

FIGURE 6 | Effect of maternal exercise on cell proliferation in the ventral DG of offspring. Representative images of Ki-67 positive cells of **(A,C)** sedentary control and **(B,D)** maternal exercise group at 20 × (scale bar = 100 μm) and 40 × (scale bar = 50 μm) magnification. **(E)** Number of proliferating cells in ventral DG of offspring from sedentary and maternal exercise group were comparable in both sexes. *n* = 5–7 per group.

the beneficial effects of maternal exercise on offspring have been consistently reported. Here, the adoption of the voluntary wheel running paradigm was motivated by a lower stressful burden that it imposes to the mothers, whereas a more stressful form of exercise, namely the forced treadmill running, was used in Dayi et al.'s (2012) study. The effects of exercise on brain development could depend on the intensity, duration, and the frequency of the exercise (Speck et al., 2014). However, in order to avoid stress caused by social isolation to pregnant moms, we adopted a paired housing condition with a shared running wheel. In doing so, we could not accurately measure the individual running activity of maternal mice during pregnancy. Since physical voluntary wheel running is performed by pregnant mom, male and female offspring from the same litter were under the influence of the same amount of running activity from the pregnant dam. It warrants further investigation on the correlations between the amount of physical activity in pregnant dams and the levels of hippocampal adult neurogenesis and behavioral phenotype in the adult male and female offspring.

In addition to the improvements in cognitive performance, we also investigated the effect of maternal exercise on the proliferation of hippocampal progenitor cells and the number of immature neurons in the adult offspring. Male offspring with maternal exercise showed an increased number of immature neurons as indicated by DCX staining in the ventral DG, whereas female offspring showed an increased number of proliferating cells as indicated by Ki-67 staining in the dorsal DG. A previous study has indicated that the ventral hippocampus is specifically responsible for modulating emotional and affective processes, whereas the dorsal hippocampus is involved in spatial learning and memory formation (Fanselow and Dong, 2010). Particularly, changes in hippocampal neurogenesis in the ventral DG could be associated with depression phenotype, as evidenced by a study showing that selective enhancement of neurogenesis in the ventral DG could reduce depression-like behavior in rodents (Banasr et al., 2006). In the present study, the results suggest that the decrease in depressive-like behavior observed in the male offspring might be partly linked to an increase in neuronal differentiation in the ventral DG. In contrast, a decrease in depressive-like behavior in females could be independent of changes in the ventral DG neurogenesis, but linked to other mechanisms. In addition to enhancement in adult neurogenesis, it is possible that maternal exercise may induce other forms of neuroplasticity leading to reduced depression-like behavior in the offspring. Animal studies have demonstrated that maternal exercise increases hippocampal levels of the brain-derived neutrophic factor (BDNF) (Liu and Nusslock, 2018), a factor important for promoting structural and functional plasticity. We have previously shown that both dendritic remodeling in the hippocampal CA3 subregion and

enhancement in adult neurogenesis in the dentate region are required for the antidepressant effects of voluntary running in a rat model of depression (Yau et al., 2011). Furthermore, running can significantly enhance long-term potentiation in the hippocampal DG in mice (van Praag et al., 1999a). Enhanced dendritic complexity and synaptic plasticity may therefore also contribute to the improvement of depression-like behavior in both male and female offspring. Also, a previous study using C57BL/6J mouse strain revealed that voluntary wheel running elicits differential effects on synaptic plasticity of male and female hippocampi (Titterness et al., 2011), warranting further investigation on whether maternal voluntary wheel running can elicit transgenerational benefits in terms of dendritic complexity and synaptic plasticity in both female and male offspring.

Studies have also shown that maternal stress during pregnancy differently affect male and female offspring in terms of hippocampal-dependent behavioral tasks, suggesting that males may be more sensitive to gestational stress than females (Bowman et al., 2004; Koenig et al., 2005; Weinstock, 2005). Similarly, our results showed that male offspring had a significant improvement in a hippocampal dependent memory, which was absent in females. It may suggest that male offspring could be more sensitive to maternal exercise than females in terms of hippocampal dependent learning and memory tasks. However, as previously stated, further studies examining the effects of different intensities of maternal running on hippocampal dependent learning and memory tasks, as well as hippocampal adult neurogenesis in female and male offspring will be of great interest. Such study will further confirm whether there is a dosage effect in response to the maternal exercise-induced increase in hippocampal neurogenesis in the offspring.

In addition, a gender-specific effect was observed with an increased number of immature neurons in the ventral DG of male offspring, and an increased number of proliferating cells in the dorsal DG of female offspring. Indeed, it has been previously shown that voluntary exercise elicits differential effects on synaptic plasticity of the male and female hippocampus (Titterness et al., 2011). It is known that sex hormones including progesterone, testosterone, and estradiol have differential effects on modulating hippocampal adult neurogenesis and dendritic complexity in male and female rodent brains (Trivino-Paredes et al., 2016). An earlier study reported that ovariectomy abrogated the exercise-induced up-regulation of BDNF mRNA level in female rats (Berchtold et al., 2001), suggesting a potential role of endogenous gonadal hormones on influencing hippocampal plasticity. Because an increase in testosterone promotes cell survival in males, but not female animals (Farinetti et al., 2015), it is possible that differences in sex hormones between males and females contribute to the sex-specific response to maternal exercise. Furthermore, we obtained the offspring from seven different litters in total. Given that there was usually a small number of pups, which were born from the first litter from the naïve dam, we only included two to four offspring from each litter for the experiment. The litter effect as a confounding factor should be considered since the litter effect could have influenced the reported results. Future studies should include two animals from each litter in a given experimental group to ensure the potential litter effects will not influence the overall results.

Despite the limitations, we have demonstrated novel findings regarding the benefits of maternal exercise on offspring and its effect on modulating hippocampal cell proliferation and neuronal differentiation of new-born cells in a sex-specific manner. Specifically, we identified differential effects of maternal voluntary wheel running on the adult offspring, with a sex-specific effect on enhancing learning and memory performance in male, but not female offspring. This behavioral benefit was partly linked to increased neuronal differentiation of new-born neurons. The current findings provide the first evidence that male and female offspring respond differently to maternal exercise. The mechanisms underlying the sex-specific effects of maternal exercise on the offspring require future studies.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because there is not a link or online drive to share the data yet at my institute. Requests to access the datasets should be directed to sonata.yau@polyu.edu.hk.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of guideline by the central animal facilities at Hong Kong Polytechnic University. The protocol was approved by the animal subjects ethics committee.

AUTHOR CONTRIBUTIONS

S-YY, LC-KL, and W-LL performed the experiments. S-YY, LC-KL, TH-YL, and DAF analyzed the data. S-YY, LC-KL, TH-YL, DAF, PS, and CC prepared the manuscript.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Traumatic Brain Injury Modifies the Relationship Between Physical Activity and Global and Cognitive Health: Results From the Barcelona Brain Health Initiative

Timothy P. Morris[1,2,3,4]*, *Jose-Maria Tormos Muñoz*[1,2,3], *Gabriele Cattaneo*[1,5,6], *Javier Solana-Sánchez*[1,2,3], *David Bartrés-Faz*[1,5,6] and *Alvaro Pascual-Leone*[1,4]

[1]Institut Guttmann, Institut Universitari de Neurorehabilitació Adscrit a la UAB, Badalona, Spain, [2]Universitat Autònoma de Barcelona, Bellaterra, Spain, [3]Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Barcelona, Spain, [4]Berenson-Allen Center for Noninvasive Brain Stimulation, Division of Cognitive Neurology, Department of Neurology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States, [5]Departament de Medicina, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona, Barcelona, Spain, [6]Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Physical activity has many health benefits for individuals with and without history of brain injury. Here, we evaluated in a large cohort study the impact of physical activity on global and cognitive health as measured by the PROMIS global health and NeuroQoL cognitive function questionnaires. A nested case control study assessed the influence of a history of traumatic brain injury (TBI) on the effects of physical activity since underlying pathophysiology and barriers to physical activity in individuals with TBI may mean the effects of physical activity on perceived health outcomes differ compared to the general population. Those with a history of TBI (n = 81) had significantly lower Global health (β = −1.66, p = 0.010) and NeuroQoL cognitive function (β = −2.65, p = 0.006) compared to healthy adults (n = 405). A similar proportion of individuals in both groups reported being active compared to being insufficiently active ($X^2_{(1)}$ = 0.519 p = 0.471). Furthermore, the effect of physical activity on global health (β = 0.061, p = 0.076) and particularly for NeuroQoL (β = 0.159, p = 0.002) was greater in those with a history of TBI. Individuals with a history of TBI can adhere to a physically active lifestyle, and if so, that is associated with higher global and cognitive health perceptions. Adhering to a physically active lifestyle is non-trivial, particularly for individuals with TBI, and therefore adapted strategies to increase participation in physical activity is critical for the promotion of public health.

Keywords: physical activity, cognition, global health, traumatic brain injury, health-related quality of life

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***Correspondence:**
Timothy P. Morris
tpmorris@bidmc.harvard.edu

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INTRODUCTION

Physical activity is associated with a 20%–30% lower risk in all-cause mortality and incidence of multiple chronic conditions (James et al., 2016). Numerous governing bodies including the World Health Organization, American Heart Association and the American College of Sports Medicine have focused much attention on the beneficial effects of a physically active lifestyle

(Warburton et al., 2006; Haskell et al., 2007; WHO, 2019a). The effects span multiple bodily systems from cardiovascular benefits (James et al., 2016) to mental health (Blumenthal et al., 1999; Babyak et al., 2000) and cognitive function (Colcombe and Kramer, 2003; Gomes-Osman et al., 2018; Northey et al., 2018). The effects of physical activity on cognitive function has received particular attention in recent decades across distinct age groups, including adolescents (Donnelly et al., 2016), older adults (Colcombe and Kramer, 2003) and patients with dementia (Gomes-Osman et al., 2018). Physical activity represents a modifiable lifestyle factor capable of improving global and cognitive health across the lifespan. However, engaging in a physically active lifestyle is non-trivial. Globally, one in four adults are classified as insufficiently active (WHO, 2019b), which is estimated to contribute to 9% of all premature deaths worldwide (or 5.3 million; Lee et al., 2012). Physical inactivity is exacerbated in those with a disability, with almost half of US adults with a disability being physically inactive and having a greater likelihood of having a chronic disease (Carroll et al., 2014).

Individuals who have acquired brain injuries can become prone to a sedentary lifestyle which places them at risk of secondary health complications (Shavelle et al., 2001). A number of studies have reported that individuals with a history of traumatic brain injury (TBI) are insufficiently active enough to receive a health benefit (Reavenall and Blake, 2010; Hamilton et al., 2015), according to current guidelines (Larry Durstine et al., 2012). The long-term health consequences of TBI include cognitive, sensorimotor, behavioral and social problems that can negatively affect quality of life (Stocchetti and Zanier, 2016), and in the US alone, an estimated 3.2 million individuals live with residual effects of TBI (Benedictus et al., 2010). Cognitive dysfunction following TBI can last for decades post-injury (Draper and Ponsford, 2008) and physical exercise is a potential therapeutic intervention for those living with residual effects of an injury (Hoffman et al., 2010; Wise et al., 2012; Chin et al., 2015). Whilst the feasibility of dedicated and professional-led, short-to-mid-term (3 months) aerobic exercise programs in community-dwelling individuals with moderate-to-severe TBI has been demonstrated (Devine et al., 2016), the association between a physically active lifestyle and global and cognitive brain health in this population has not been assessed. Distinct barriers to performing physical activity (Rimmer et al., 2004) and underlying pathophysiology (Werner and Engelhard, 2007) may mean that this relationship differs in individuals with a history of TBI.

We performed a nested case-control study to assess the impact of a history of TBI on the associations between physical activity and perceived global and cognitive health. We hypothesized that physical activity would be predictive of higher global and cognitive health in both those with and without a history of TBI.

MATERIALS AND METHODS

Study Design

A cohort of community-dwelling adults, mainly in the Catalonia region of Spain, was established as part of the Barcelona Brain Health Initiative[1], starting in 2017. Adults aged 40–65 were invited to participate in an online questionnaire-based survey *via* television and local advertisements. The study protocol is described in Cattaneo et al. (2018). All methods described were approved by the education and ethics committee of Institut Guttmann. This manuscript was prepared under the strengthening the reporting of observational studies in epidemiology (STROBE) guidelines.

At the time of analysis (June 2018), a total of 4,624 individuals had enrolled. Of those, 81 individuals (1.8%) answered positively to the question: *Have you ever had a TBI with loss of consciousness?* A control group (N = 405, 49% female) was randomly selected from the total cohort using Microsoft Excel's "rand" function. The total cohort was organized by this random number sequence and then filtered per the age and gender criteria. Whereby five adults free from any neurological or psychological disorders were selected for every participant with a history of TBI at random in blocks of 5 years with an even male to female ratio.

Outcomes and Covariates

Our main outcome variables were perceived global health and perceived cognitive function. To measure these constructs, the PROMIS global health questionnaire (Cella et al., 2010) and the NeuroQoL cognitive function questionnaire (Gershon et al., 2012) were used. PROMIS global health is a 10-item 5-point Likert scale (1-poor, 5-excellent) that probes respondents physical, mental and social health. Higher scores mean more of the construct is being measured (i.e., higher global health). NeuroQoL cognitive function is 5-point Likert scale (1-very often, 5-never) with 12 items that probes respondents thinking, attention, planning, new task learning and comprehension. Higher scores mean more of the construct is being measured (i.e., better perceived cognitive function).

Our main predictor variable was physical activity levels. We chose to implement the Godin-Shepard leisure time physical activity questionnaire (Godin and Shephard, 1985) to measure this. The GSLTPAQ probes the number of times spent performing moderate (not exhausting) or strenuous (heart beats rapidly) physical activity of at least 15-min during a typical 7-day period (Godin, 2011). The frequency score is multiplied by a corresponding metabolic equivalent for task (MET) value (moderate = *5; strenuous = *9) and summed to obtain an arbitrary leisure score index (LSI). Cut-off points can be created using the LSI, the rationale for which originate from the World Organization (WHO, 2018) and American College of Sports Medicine (Ferguson, 2014) guidelines for weekly physical activity associated with significant health benefits (combination of moderate and strenuous activity 3–5 times per week). An LSI of $\geq$24 is *active* where as those $\leq$23 are *insufficiently active*. Consequently, those culminating in a score of $\geq$24 using questions that pertain to moderate and strenuous physical activity and LSI calculations based on both frequency and energy expenditure will likely meet the physical activity guidelines. The utility and accuracy of these cut-off scores have been validated

[1] www.bbhi.cat

in healthy adults (Amireault and Godin, 2015). Raw scores above 7 for each question were excluded (N = 13) as they were considered to be derived through misinterpretation of the question. Those without responses were set to missing (N = 12).

Co-variates included gender and age, which was asked in years and three categories were created; 40–49, 50–59 and 60 and above. BMI was calculated as body weight (in kilograms) divided by height (in meters squared). Self-perceived negative affect in depression, anxiety and stress was also assessed using the 21-item sub-scale version of the Depression Anxiety Stress Scale (Brown et al., 1997). This is a 4-point Likert scale (1-never, 4-always) where higher scores represent higher negative affective state. This was chosen as a potential confounder as perceived negative effect can affect how one reports cognitive and global health (Bartrés-Faz et al., 2018).

Statistical Analysis

We tested age, gender and negative affective status as potential confounding variables in our analysis. Variables which predicted the outcome measure with a $p \leq 0.05$ were defined as confounders and added to the final model. The proportion of individuals who reported being insufficiently active compared to active was tested using Pearson's chi squared test of proportions, as was gender. A Kruskal-Wallis test was used to assess differences in BMI between groups. Generalized linear models with a Gaussian family function and identity link function were used to assess the independent associations between physical activity levels and diagnosis history (history of TBI or not) on perceived cognitive function and perceived global health, controlling for all other significant variables in the model. To assess whether the associations between physical activity and perceived cognitive and global health differed between those with and without a history of TBI, an interaction term between physical activity and diagnosis history was added to the model. Post estimation tests were performed using marginal effects and the estimated slopes were plotted. All statistical analyses were performed in Stata version 15 (StataCorp LLC, College Station, TX, USA).

RESULTS

Table 1 describes all participant characteristics and questionnaire scores for each group. There was no significant difference between the age of the participants in each group ($t_{(116)} = -0.3$, $p \geq 0.740$) and the proportion of male and female participants was similar across groups [49% female ($X^2_{(1)} = 0.004$ $p = 0.951$)]. BMI was not significantly different between each group ($X^2_{(1)} = 0.042$ $p = 0.838$).

Physical Activity Level

Using the LSI cut-off scores to classify participants into *active* and *insufficiently active*, a similar proportion of those with and without and history of TBI were classified as *active* ($X^2_{(1)} = 0.519$ $p = 0.471$), compared to *insufficiently active* (**Figure 1**).

PROMIS Global Health

The distribution of Promis global health scores in both groups is shown in **Figure 2A**. Diagnosis was a significant predictor of global health ($\beta = -1.66$, SE = 0.644, $p = 0.010$, 95% CI's = −2.927, −0.403) with those with a history of TBI reporting lower perceived global health (32.2 ± 0.70) than those without (34.9 ± 0.22). Physical activity level significantly predicted Promis global health ($\beta = 0.049$, SE = 0.011, $p \leq 0.001$, 95% CI's = 0.027, 0.071). A non-significant interaction between diagnosis and physical activity level was shown ($\beta = 0.061$, SE = 0.034, $p = 0.076$, 95% CI's = −0.006, 0.128). Post estimation tests showed that the relationship between physical activity and global health was marginally greater in the group with a history of TBI ($\beta = 0.103$, SE = 0.032, $p = 0.001$, 95% CI's = 0.040, 0.166), than in the healthy adults ($\beta = 0.042$, SE = 0.011, $p \leq 0.001$, 95% CI's = 0.019, 0.065; **Figure 3A**).

TABLE 1 | Participant characteristics and questionnaire scores between groups.

	Healthy adults	History of TBI
Age (years)	51.8 (7.2)	51.7 (7.1)
BMI (kg/m^2)	24.2 (3.5)	24.4 (4.0)
PROMIS Global Health	34.9 (4.6)	32.2 (6.2)*
PROMIS NeuroQoL	52.3 (6.5)	47.5 (11.0)*
DASS21	13.4 (11.3)	20.4 (17.5)*
GSLTPAQ	19.5 (19.2)	18.8 (18.3)

Significant at $p < 0.05$. GSLTPAQ, Godin-Shepard leisure time physical activity questionnaire.

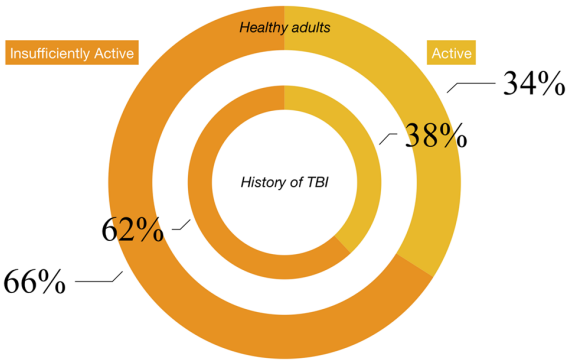

FIGURE 1 | Hollow pie charts show the proportion of individuals who report being active (yellow bars) compared to insufficiently active (orange bars) based on the Godin-Shepard leisure time physical activity questionnaire (GSLTPAQ). The Godin questionnaire classifies people as *active* or *insufficiently active* based on the number of times per week spent performing either moderate or strenuous physical activity of at least 15 min. The inner bars represent the group with a history of traumatic brain injury (TBI) and the outer bars represent those free from any neurological or neuropsychiatric disease. A numerically higher proportion of individuals in both groups report being *insufficiently active* compared to those reporting being *active*.

NeuroQoL Cognitive Function

The distribution of NeuroQoL cognitive function scores in both groups is shown in **Figure 2B**. Diagnosis was a significant predictor of NeuroQoL cognitive function ($\beta = -2.65$, SE = 0.974, $p = 0.006$, 95% CI's = −4.561, −0.743), with those with a history of TBI reporting lower perceived cognitive function health (47.45 ± 1.20) than those without (52.26 ± 3.29). Physical activity level was predictive of NeuroQoL cognitive

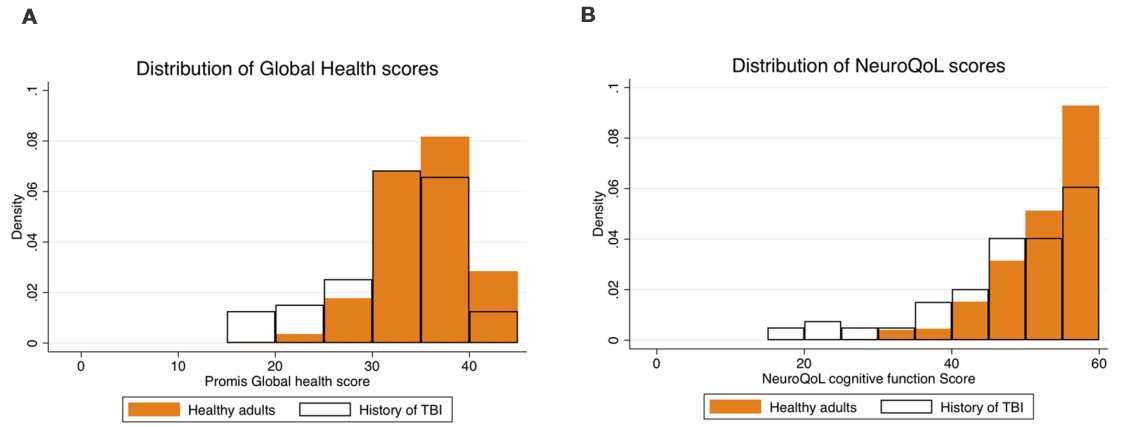

FIGURE 2 | Histogram plots show the distribution of scores on the global health scale **(A)** and the NeuroQoL cognitive function scale **(B)** for both participants in the healthy group (orange filled bars) and the group with a history of TBI (black outlined bars).

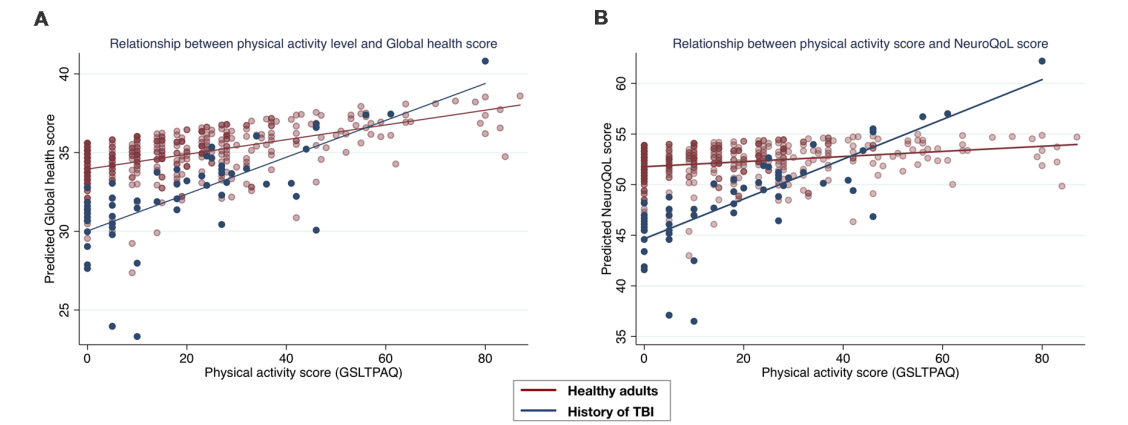

FIGURE 3 | Scatter plots of the relationship between physical activity levels and the predicted Global health and NeuroQoL cognitive function scores as a result of the generalized linear models, by group. **(A)** A trending interaction (p = 0.076) was observed between groups whereby the group with a history of TBI had a greater effect of physical activity level on global health (navy line). In **(B)** a significant interaction between groups is observed (p = 0.002) whereby the group with a history of TBI shows a significant effect of physical activity on NeuroQoL cognitive function, which is not significant for the group without a history of TBI (red line).

function (β = 0.037, SE = 0.016, $p \leq$ 0.023, 95% CI's = 0.005, 0.070). A significant interaction between diagnosis and physical activity level was shown (β = 0.159, SE = 0.051, p = 0.002, 95% CI's = 0.058, 0.260) whereby post estimation tests showed that the relationship between physical activity and NeuroQoL cognitive function was significant in the group with a history of TBI (β = 0.179, SE = 0.049, $p \leq$ 0.001, 95% CI's = 0.083, 0.274), but not in the healthy adults (β = 0.019, SE = 0.017, p = 0.260, 95% CI's = −0.014, 0.054; **Figure 3B**).

DISCUSSION

In this study, we aimed to assess the impact of a history of TBI on the relationship between physical activity levels and global and cognitive brain health. Not surprisingly, we found that healthy adults reported higher global and cognitive brain health compared to those with a history of TBI. However, we also found that individuals with a history of TBI were as likely to be physically active as those without a history of TBI and physical activity levels were even stronger predictors of both global and cognitive function health perceptions than in individuals without a history of TBI.

Self-reported levels of physical activity have been associated with numerous protective health benefits such as reduced risk of cognitive impairment (Laurin et al., 2001) and cognitive decline (Sofi et al., 2011), mortality due to cardiovascular disease (Nocon et al., 2008) and reduced incident rates of dementia (Larson et al., 2006). Self-report physical activity has also been associated with better health-related quality of life (HRQOL; Brown et al., 2003). These findings from the 2001 behavioral risk factor surveillance system survey found that adhering to recommended levels of physical activity was significantly associated with less days of poor perceived mental and physical health. Our results corroborate these findings in so much as higher physical activity

levels are related to higher perceptions of global (mental, physical, social and pain) health. Thereupon, physical activity appears not only associated with improved health outcomes but with higher perceptions of health also.

Numerous randomized-control trials have shown positive associations between physical activity and physical exercise and cognitive function (Colcombe and Kramer, 2003; Gomes-Osman et al., 2018). Additionally, a number of these studies have also shown improvements in the structure and function of cortical networks associated with cognitive functioning (Erickson et al., 2011; Voss et al., 2013; Weng et al., 2017). Whilst dose-response studies are limited (Vidoni et al., 2015; Chen et al., 2018), a linear relationship between physical activity and health status has been reported, such that increases in physical activity leads to greater health benefits, especially in previously sedentary individuals (Warburton et al., 2006). Our results, however, failed to show any association between physical activity levels and perceptions of cognitive functioning in middle to older aged adults free from any neurological or neuropsychiatric disease. This may be explained by the difference in the characteristics of the included participants in our analysis whereby those with a history of TBI had significantly lower NeuroQoL scores compared to those without a history of TBI. This could have been further exacerbated by a ceiling effect in the NeuroQoL scale for the healthy adult population. This scale was developed to assess cognitive complaints in those with neurological afflictions and so may fail to capture a large enough variance in cognitive health perceptions amongst healthy adults (Gershon et al., 2012). Additionally, perceptions of cognitive function may not correlate well with objective measures of cognitive functioning, which is the case in a number of clinical populations (Schiehser et al., 2011; Hutchinson et al., 2012). Consequently, whilst it may be the case that those with lower perceptions of cognitive health receive a greater benefit of adhering to a physically active lifestyle, studies with objective measures of cognitive functioning may further help delineate this relationship.

Some concepts of HRQOL in TBI overlap with those of the general population yet research suggests that HRQOL following TBI may be more complex (Carlozzi et al., 2011). We saw that individuals with a history of TBI had significantly lower self-reported global and cognitive brain health compared to neurologically healthy adults. Whilst we cannot be certain that this lower perception of global and cognitive brain health is derived from the injury, previous reports have shown many individuals with a history of TBI live with residual negative effects of the injury (Benedictus et al., 2010). Cognitive dysfunction is prevalent post-injury and deficits can be seen at 6 months (Dikmen et al., 2009) and for as long as 10 years after injury (Draper and Ponsford, 2008). Long-term lifestyle interventions aimed at reducing these deficits are therefore of great importance to those living with residual effects of TBI.

We found that a similar proportion of those adults with a history of TBI reported being active as those without a history of TBI. However, a numerical majority of participants (both in the history of TBI and healthy group) were classified as insufficiently active. Previous research has suggested that those with a disability or a TBI have distinct barriers to engaging in physical activity (Rimmer et al., 2004; Reavenall and Blake, 2010; Pinto et al., 2018), including but not limited to having health concerns and lack of counselling by a physician (Pinto et al., 2018). Therefore, strategies to increase adherence to a physically active lifestyle, such as the WHO's Global Strategy on Diet and Physical Activity (Bauman and Craig, 2005), are not only critical for both the general population and community-dwelling adults with a history of TBI but may need to be adapted to those living with a history of TBI. Promising results from a feasibility study of aerobic exercise programs in community-dwelling individuals with a history of moderate-to-severe TBI showed that greater adherence to exercise was achieved when free access to local gymnasiums was provided (Devine et al., 2016). Our results suggest that strategies like these that will increase adherence to a physically active lifestyle, will likely lead to improved perceptions of global and cognitive health.

Our study has certain limitations that may limit the interpretation of the results. Self-reported health outcomes, specifically TBI can be problematic and whilst sports concussion research has improved the self-reporting of concussion through better descriptions of concussion definitions (Robbins et al., 2014), this type of description is less well defined for the general public. Though the accuracy of self-reported measures of certain health outcomes (including stroke) have been documented (Okura et al., 2004), self-reported measures are often the best tool available, especially for large cohort studies. Given this constraint, we did not assess the severity of an individual's TBI nor the time since injury in our cohort of TBI. Whilst this should not affect the exposure/outcome relationship, it means that we cannot be certain whether different injury severities are more or less associated with the results found. This might be of interest to future studies. Additionally, if co-morbid medical conditions were reported in the history of TBI group, they were not excluded from the analysis. Nevertheless, the number of participants in this group who had multiple conditions was small (headache (Lee et al., 2012), chronic pain (Babyak et al., 2000), anxiety (Gomes-Osman et al., 2018), depression (Blumenthal et al., 1999), memory loss (James et al., 2016), heart attack (Warburton et al., 2006), sleep apnea (Haskell et al., 2007), arthritis (James et al., 2016) and so are unlikely to have significantly affected the results.

CONCLUSION

Adhering to a physically active lifestyle is associated with higher global and cognitive health perceptions, especially in individuals with a history of TBI. Notwithstanding, a majority of individuals are insufficiently active and therefore it is critical to develop strategies to increase adherence to and participation in a physically active lifestyle in both those with and without a history of TBI.

DATA AVAILABILITY

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

any qualified researcher. The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethics and education committee of Institut Guttmann with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "Institut Guttmann."

AUTHOR CONTRIBUTIONS

AP-L, DB-F and J-MTM performed the initial conception of the project. GC and JS-S collected the data. TM analyzed the data. TM drafted the manuscript and all authors critically revised it for intellectual content.

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Conflict of Interest Statement: AP-L serves on the scientific advisory boards for Nexstim, Neuronix, Starlab Neuroscience, Neuroelectrics, Constant Therapy, Cognito, and Neosync, and is listed as an inventor on several issued and pending patents on the real-time integration of transcranial magnetic stimulation with electroencephalography and magnetic resonance imaging.

The remaining 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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Effects and Mechanisms of Cognitive, Aerobic Exercise, and Combined Training on Cognition, Health, and Brain Outcomes in Physically Inactive Older Adults: The Projecte Moviment Protocol

*Alba Castells-Sánchez[1,2,3], Francesca Roig-Coll[1], Noemí Lamonja-Vicente[1,2,3], Marina Altés-Magret[1], Pere Torán-Monserrat[4], Marc Via[1,2,3], Alberto García-Molina[5], José Maria Tormos[5], Antonio Heras[4], Maite T. Alzamora[4], Rosa Forés[4], Guillem Pera[4], Rosalia Dacosta-Aguayo[1†], Juan José Soriano-Raya[1,2], Cynthia Cáceres[6], Pilar Montero-Alía[4], Juan José Montero-Alía[4], Maria Mercedes Jimenez-Gonzalez[4], Maria Hernández-Pérez[6], Alexandre Perera[7], George A. Grove[8], Josep Munuera[9], Sira Domènech[10], Kirk I. Erickson[8] and Maria Mataró[1,2,3]**

[1] Department of Clinical Psychology and Psychobiology, University of Barcelona, Barcelona, Spain, [2] Institut de Neurociències, University of Barcelona, Barcelona, Spain, [3] Institut de Recerca Pediàtrica, Hospital Sant Joan de Déu, Barcelona, Spain, [4] Unitat de Suport a la Recerca Metropolitana Nord, Fundació Institut Universitari per a la Recerca a l'Atenció Primària de Salut Jordi Gol i Gurina, Mataró, Spain, [5] Institut Guttmann, Institut Universitari de Neurorehabilitació, Universitat Autònoma de Barcelona, Barcelona, Spain, [6] Department of Neurosciences, Hospital Universitari Germans Trias i Pujol, Barcelona, Spain, [7] B2SLab, Departament d'Enginyeria de Sistemes, Automàtica i Informàtica Industrial, Universitat Politècnica de Catalunya, Barcelona, Spain, [8] Department of Psychology, University of Pittsburgh, Pittsburgh, PA, United States, [9] Diagnostic Imaging Department, Fundació de Recerca, Hospital Sant Joan de Déu, Barcelona, Spain, [10] Institut de Diagnòstic per la Imatge, Hospital Universitari Germans Trias i Pujol, Barcelona, Spain

Introduction: Age-related health, brain, and cognitive impairment is a great challenge in current society. Cognitive training, aerobic exercise and their combination have been shown to benefit health, brain, cognition and psychological status in healthy older adults. Inconsistent results across studies may be related to several variables. We need to better identify cognitive changes, individual variables that may predict the effect of these interventions, and changes in structural and functional brain outcomes as well as physiological molecular correlates that may be mediating these effects. Projecte Moviment is a multi-domain randomized trial examining the effect of these interventions applied 5 days per week for 3 months compared to a passive control group. The aim of this paper is to describe the sample, procedures and planned analyses.

Methods: One hundred and forty healthy physically inactive older adults will be randomly assigned to computerized cognitive training (CCT), aerobic exercise (AE), combined training (COMB), or a control group. The intervention consists of a 3 month home-based program 5 days per week in sessions of 45 min. Data from cognitive, physical, and psychological tests, cardiovascular risk factors, structural and functional brain scans, and blood samples will be obtained before and after the intervention.

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****Correspondence:***
Maria Mataró
mmataro@ub.edu

†Present address:
Rosalia Dacosta-Aguayo, Center for Neuropsychology and Neurosciences Research, Kessler Foundation, East Hanover, NJ, United States

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Results: Effects of the interventions on cognitive outcomes will be described in intention-to-treat and per protocol analyses. We will also analyze potential genetic, demographic, brain, and physiological molecular correlates that may predict the effects of intervention, as well as the association between cognitive effects and changes in these variables using the per protocol sample.

Discussion: Projecte Moviment is a multi-domain intervention trial based on prior evidence that aims to understand the effects of CCT, AE, and COMB on cognitive and psychological outcomes compared to a passive control group, and to determine related biological correlates and predictors of the intervention effects.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier NCT03123900.

Keywords: computer-based cognitive training, aerobic exercise, neuroplasticity, neuroimaging, biomarkers, physically inactive, aging, fitness

INTRODUCTION

Walk, learn, be active, do! A large number of messages about healthy behaviors to reduce age-related functional decline has flooded into our daily lives. Aging is related to major risk of cardiovascular diseases, metabolic syndrome, mitochondrial dysfunction, obesity, sarcopenia, and consequent higher inflammation, oxidative stress, and brain and cognitive impairment (Sallam and Laher, 2016). Healthy aging has become a matter of interest for the scientific community and for most people and governments that stand for social health policies. Since the aged population is expected to triple by 2100 and will represent 29% of people in the world (United Nations Department of Economic Social Affairs Population Division, 2017), we need policies and strategies that enhance independence and quality of life while considering economic, social, environmental, and personal determinants as well as health and social services (World Health Organization [WHO], 2002, 2015). Cognitive training and aerobic exercise are two lifestyle interventions that have proved to produce positive effects on health (Cotman et al., 2007; Sallam and Laher, 2016), reduce cognitive impairment (Harada et al., 2013), and delay the onset of dementia (Hall et al., 2009). However, questions about which, when and why remain unclear.

Gates and Valenzuela (2010) define cognitive training as an intervention consisting of repeated practice of standardized exercises targeting a specific cognitive domain or domains. Computerized cognitive training (CCT) has emerged as a new tool to systematically apply these exercises. CCT facilitates the administration by allowing investigators to adapt the content and challenge of the task to individual performance and including visual engaging interfaces (Lampit et al., 2014; Shao et al., 2015). There is evidence that CCT may maintain or improve global cognitive function and specific trained functions such as verbal memory (Shao et al., 2015; Barban et al., 2016; Bahar-Fuchs et al., 2017), processing speed (Kueider et al., 2012; Lampit et al., 2014; Shao et al., 2015), and executive function (Kueider et al., 2012; Barban et al., 2016). Brain related benefits such as increases in gray matter volume of default-mode network (DMN) areas (De Marco et al., 2016), functional activity of frontal-parietal networks (Klingberg, 2010; Jolles et al., 2013; Kim et al., 2017) and connectivity of the hippocampus (Lisanne et al., 2017) and posterior DMN (De Marco et al., 2016) have also been described. These structural and functional changes appear to be directly related to the types of trained tasks (Taya et al., 2015). Despite this, the biological pathways by which CCT produces these effects remain poorly understood in humans. Shao et al. (2015) hypothesized that these mechanisms might be related to brain neuroplasticity. According to Hebb (1949), a group of neurons that are repeatedly and simultaneously activated will tend to form stronger associations. This framework suggests that CCT may influence cognition by promoting the strength of synaptic connections (Patterson et al., 1996; Taya et al., 2015). Based on animal models, Valenzuela and Sachdev (2009) suggested that brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) might be the molecules promoting cell survival and proliferation after cognitive stimulation in humans.

Physical activity (PA), defined as any body movement produced by skeletal muscles that results in energy expenditure (Caspersen et al., 1985), promotes health, cognitive and psychological benefits (DiLorenzo et al., 1999; Penedo and Dahn, 2005). Exercise, which is considered a planned, structured and repetitive subtype of PA that aims to improve physical fitness (Caspersen et al., 1985), produces an acute body reaction that includes increased energy expenditure, repetitive muscle contractions and an inflammatory and oxidative response (van Praag et al., 2014; Sallam and Laher, 2016). Different types of exercise, applied in a regular manner, may produce different physiological, brain and cognitive benefits (Barha et al., 2017; Cabral et al., 2019). Several systematic reviews conclude that aerobic exercise (AE), the type of exercise that involves oxygen consumption and movement of large groups of skeletal muscles during a sustained period of time (Chodzko-Zajko et al., 2009; Thomas et al., 2012), may improve executive function, processing speed, attention and memory in healthy older adults (Etnier et al., 1997; Colcombe and Kramer, 2003; Paterson and Warburton, 2010; Smith et al., 2010; Guiney and Machado, 2012; Karr et al., 2014; Scherder et al., 2014; Lü et al., 2016; Barha et al., 2017; Northey et al., 2017). However, other reviews reported that the evidence was too limited to draw firm conclusions

(Snowden et al., 2011; Cox et al., 2016; Brasure et al., 2017; Sáez de Asteasu et al., 2017) or reported no significant effects of exercise on cognition (Angevaren et al., 2007; Kelly et al., 2014; Young et al., 2015). Regular AE has direct effects on our body: higher oxygen and glucose consumption related to increased energy expenditure, and reduction of body fat and increased muscle strength, which have been hypothesized as specific pathways for the physiological relationship between exercise and cognitive function (Cotman et al., 2007; van Praag et al., 2014; Sallam and Laher, 2016; Stimpson et al., 2018). The increase of energy expenditure reduces visceral fat that may lead to less production of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha) and an increase of M2:M1 macrophage ratio and the release of adiponectin. Energy expenditure is also related to higher glucose consumption which may be related to better energy metabolism and insulin sensitivity and reducing resistance to leptin and insulin (van Praag et al., 2014). The activity in the muscles induces IL-ra, IL-10, and heat shock proteins (HSP), reducing the inflammatory environment while suppressing IL-1 and TNF-alpha and upregulating IL-15 and promoting the reparation of the vessels to facilitate blood flow and, as a consequence, oxygen and nutrient circulation (Sallam and Laher, 2016). Skeletal muscles may also improve the use of lipids instead of glycogen in energy expenditure processes. Exercise increases circulating HDL and reverses cholesterol transport, reducing cholesterol levels in blood (Mann et al., 2013). The activity in the cardiovascular system produces laminar shear stress on vascular endothelial cells which may be related to the downregulation of oxidative processes, and activates the hypothalamic-pituitary-adrenal axis which triggers the release of glucocorticoids that may help to inhibit the inflammatory system. The anti-oxidative response is mediated by redox-sensitive transcription factors: NF-KB and AP-1, which reduce RONS, and PGC-1, which promotes mitochondrial biogenesis (Sallam and Laher, 2016). Laminar shear stress is also related to greater release of insulin growth factor (IGF) and vascular endothelial factor (VEGF) which benefits the cardiovascular system, helping to repair the body vasculature and promoting greater blood flow, brain angiogenesis and neurogenesis (Cotman et al., 2007; Sallam and Laher, 2016; Stimpson et al., 2018; Cabral et al., 2019). IGF promotes the release of BDNF in the brain, which has been identified as one of the principal factors mediating the effect of exercise on cognition. BDNF may support newborn cells, regulate synaptic changes and facilitate long-term potentiation which may be related to the identified brain changes and cognitive benefits (Stimpson et al., 2018; Cabral et al., 2019). Cardiorespiratory fitness (CRF), the health-related component of physical fitness reflecting these parameters, has shown to be related to better cognitive function in healthy adults (Colcombe and Kramer, 2003). However, Etnier et al. (2006) and Young et al. (2015) could not find the relationship between changes in CRF and changes in cognition in their systematic reviews. Erickson et al. (2014) found a positive relationship between PA or CRF and gray matter volume in older adults in prefrontal, temporal and parietal areas (Erickson et al., 2007; Gordon et al., 2008; Weinstein et al., 2012). Higher levels of CRF have been also related to greater hippocampus volume and memory performance (Erickson et al., 2009; Szabo et al., 2011) and bigger caudate nucleus and nucleus accumbens (Verstynen et al., 2012). However, Rosano et al. (2010) and Smith et al. (2011) did not find a significant association between PA and gray matter volume. Sexton et al. (2016) systematically reviewed the effects of exercise on white matter volume – global, local, lesions, and microstructure – and found cautious support for this association given the fact that evidence was inconsistent. Recent research aims to identify the effect of exercise on functional connectivity. CRF has been associated with higher general efficiency and lower local efficiency and executive function performance (Kawagoe et al., 2017). Brain network modularity at baseline may predict the effects of exercise intervention (Baniqued et al., 2018). Other variables have been identified as potential modifiers of the association between exercise and cognition. Groups with a higher percentage of women (Barha and Liu-Ambrose, 2018) or APOE E4 genotype carriers (Etnier et al., 2007) may benefit more from exercise.

The combination (COMB) of PA and cognitive stimulation may induce greater cognitive benefits compared to each intervention separately (Kraft, 2012; Curlik and Shors, 2013; Fissler et al., 2013; Bamidis et al., 2014; Law et al., 2014; Lauenroth et al., 2016). However, Shatil (2013) found improvements only on those participants engaged in cognitive training, single or combined. Zhu et al. (2016) replicated these results in a systematic review of twenty studies, concluding that COMB may have a small positive effect only when compared to a control and physical activity group but not to a cognitive intervention. To our knowledge, the specific cognitive benefits of COMB, in sequence or dual task, remain unknown; undefined "greater effects" or "more enduring" are usually hypothesized. General cognitive function (Oswald et al., 2006; Shatil, 2013), executive function (Anderson-Hanley et al., 2012; Theill et al., 2013; Barcelos et al., 2015; Eggenberger et al., 2015), processing speed (León et al., 2015), memory (Fabre et al., 2002) and vocabulary (Schmidt-Kassow et al., 2013) performance may tend to benefit more from a COMB. However, evidence is not consistent across trials and negative results have also been found in these same domains (Fabre et al., 2002; Oswald et al., 2006; Legault et al., 2011; Anderson-Hanley et al., 2012; Linde and Alfermann, 2014; Rahe et al., 2015). Li et al. (2014) and Pieramico et al. (2012) reported that a multimodal intervention produced a reorganization of functional connectivity between the DMN areas. Shah et al. (2014) identified higher verbal memory related to increased glucose metabolism in the brain in the COMB group only. In order to explain these potential greater benefits, Olson et al. (2006) and Fabel et al. (2009), based on animal models, hypothesized that neuroplasticity may be facilitated by exercise and guided by cognitive training. The anti-inflammatory, anti-oxidative stress and cardiovascular and neural repairing responses related to regular PA may enhance cell proliferation through BDNF. Cognitive stimulation may promote the survival of newborn cells and regulate synaptic changes (Hebb, 1949).

Systematic reviews and papers cited before, independently of the intervention, reported inconsistencies across results which likely relate to genetic and environmental variables of

participants; type, duration and schedule of assessments; type, duration, frequency, intensity and adherence of interventions; as well as methodological issues of the design, such as type of control group and statistical approaches (Kraft, 2012; Young et al., 2015; Gates et al., 2019). These discrepancies challenge a clear theoretical model and lead to different conclusions and the identification of potential moderators even at the systematic and meta-analytic analysis level. These issues highlight the need to better identify not only the cognitive effects of these interventions but also the individual variables that may predict them and the brain changes and physiological molecular correlates that may be mediating any benefits.

Projecte Moviment is a multi-domain randomized trial that addresses the effect of CCT, AE, and COMB on cognition and psychological status in healthy physically inactive older adults compared to a passive control group. We also aim to identify variables that may predict the effects of the intervention and the underlying brain changes and physiological molecular correlates that may mediate the effects. The purpose of this paper is to describe the protocol in accordance with SPIRIT Guidelines.

AIMS OF THE STUDY

The primary objective of Projecte Moviment is to examine the effect of CCT, AE, or COMB on cognitive outcomes in healthy physically inactive older adults. The primary hypotheses sustaining this goal are:

1. Computerized cognitive training – 5 times per week for 3 months – will improve general cognitive function as well as trained cognitive functions (executive function, processing speed and memory) measured by composite scores using a battery of validated neuropsychological tests at 3 months compared to a control group.
2. Aerobic exercise – 5 times per week for 3 months – will improve executive function, attention-processing speed and memory measured by composite scores using a battery of validated neuropsychological tests at 3 months compared to a control group.
3. Combined training – 5 times per week for 3 months – will show greater improvements in general cognitive function, executive function, attention-processing speed and memory measured by composite scores using a battery of validated neuropsychological tests at 3 months compared to a control group.

The secondary objectives of Projecte Moviment are: (a) to determine the effects of these interventions on psychological status and subjective performance on daily activities, CRF, brain structure and function and physiological molecular correlates; (b) to identify genetic, demographic, physiological and brain variables that might predict the effect of the intervention; (c) to identify the association between cognitive effects and other psychological, physiological correlates. Specific hypotheses for each objective will be specified in each article when reporting results. General secondary hypotheses include:

1. All intervention conditions will positively impact psychological and subjective daily functional performance assessed by questionnaires compared to controls.
2. Aerobic exercise and COMB will similarly increase CRF and energy expenditure in daily activity compared to cognitive and control conditions.
3. All intervention conditions will positively impact the structure and function of the brain assessed by whole brain analyses, structures of interest and white matter lesions volume and microstructure, cortical thickness and functional connectivity compared to a control group.
4. Aerobic exercise and COMB will improve immunity, reduce inflammation and improve vascular risk factors compared to cognitive and control conditions.
5. Individual variables (i.e., sex, age, cognitive baseline, CRF baseline) will predict the effect of the interventions on cognition.
6. Changes in cognition will be related to specific changes in secondary outcomes depending on the intervention.

METHODS

Study Oversight and Schedule

Projecte Moviment is a multi-center, single-blind randomized controlled trial that started November 2015 with the aim of recruiting 140 participants distributed in four parallel groups (one control group, $n = 20$; three intervention groups, $n = 40$ each). All participants give their written informed consent and are assessed at baseline and 3 months later, immediately after the intervention (**Figure 1**). This study is led by the Faculty of Psychology of the University of Barcelona in collaboration with Institut Universitari d'Investigació en Atenció Primària Jordi Gol, Hospital Germans Trias i Pujol and Institute Guttmann; it was approved by the responsible ethics committees following the Declaration of Helsinki.

Participants

Participants are 140 community dwelling physically inactive healthy adults aged 50–70 years from Barcelona. Inclusion and exclusion criteria are detailed in **Table 1**. Multiple strategies are applied to recruit participants: distribution of posters and flyers, publication of press releases in local media (newspapers, radio, and TV), presentations in local community organizations, list of patients of general physicians and volunteers from previous studies. Participants are enrolled in primary care centers and can voluntarily withdraw from the project at any time.

Assessments

Potential participants are screened by phone and an on-site personal interview; if eligible, informed written consent is obtained. Assessments are conducted in a clinical environment and organized into three appointments that take place at baseline – within 2 weeks prior to the start of the intervention, and again at 3 months within 2 weeks after the completion of the intervention (**Table 2**). (1) Medical assessment (30 min): review of medical history and current health status including

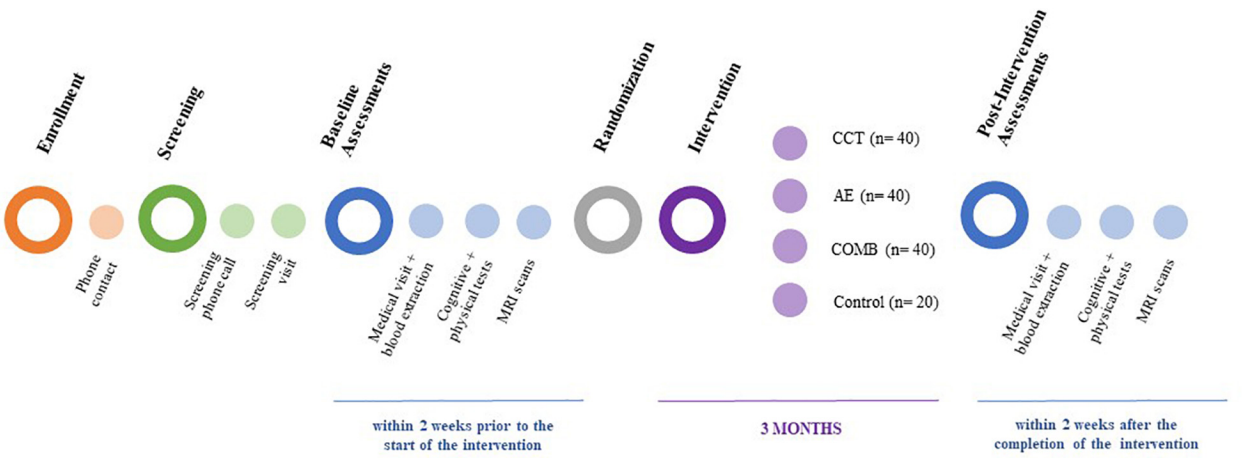

FIGURE 1 | Projecte Moviment study design. CCT, computer-based cognitive training; AE, aerobic exercise; COMB, combined training.

TABLE 1 | Inclusion and exclusion criteria for Projecte Moviment.

Inclusion criteria	Exclusion criteria
Aged 50–70 years	Current participation in any cognitive training activity or during last 6 months > 2 h/week
≤120 min/week of physical activity during last 6 months	Diagnostic of dementia or mild cognitive impairment
Mini-Mental State Examination (MMSE) ≥ 24	Diagnostic of neurological disorder: stroke, epilepsy, multiple sclerosis, traumatic brain injury, brain tumor
Montreal Cognitive Assessment 5-min (MoCA 5-min) ≥ 6	Diagnostic of psychiatric illness current or during last 5 years
Competency in Catalan or Spanish	Geriatric Depression Scale (GDS-15) > 9
Adequate visual, auditory, and fine motor skills	Consumption of psychopharmacological drugs current or during last 5 years; or more than 5 years throughout life
Acceptance of participation in the study and signature of the informed consent	History of drug abuse or alcoholism current or during last 5 years; or more than 5 years throughout life; >28 men and >18 woman unit of alcohol/week
	History of chemotherapy
	Contraindication to magnetic resonance imaging

MMSE (Blesa et al., 2001); MoCA 5-min (Wong et al., 2015); GDS-15 (Martínez et al., 2002).

cardiovascular risk factors and blood extraction between 8 and 9:30 AM following an overnight fast. (2) Cognitive assessment and physical status (2.5 h): administration of a battery of neuropsychological tests, psychological health, subjective performance, daily activities and PA questionnaires and a treadmill fitness test. In order to control the effects of acute exercise, participants are advised not to exercise prior to all appointments and cognitive tests are conducted before the CRF test. (3) Magnetic Resonance Imaging (MRI) scans (45 min): administration of a neuroimaging protocol to acquire structural and functional data of the brain. All assessments are carried out in two primary care centers except MRI scans that are performed in the Hospital Germans Trias i Pujol. All subjects receive an actimeter the first and last week of intervention to track their daily activity and determine if participants meet the intervention protocol. The same team of psychologists, which administers cognitive tests, and nurses, who collect general health data, follow the protocol in all the centers.

Randomization

Participants are randomly assigned to the groups stratified by sex, age, and education. The allocation sequence consists of a random list of these variables all combined and was generated by a statistician. The intervention staff is responsible for the allocation and informs participants at the first intervention visit. Assessors are blinded to the sequence and the group assignment of the participants. Blinding will only be broken for medical reasons.

Intervention

The intervention consists of a 3 months program, 5 days per week in sessions of 45 min. At baseline all participants receive oral and written information about their specific intervention, an actimeter with brief instructions, and a follow-up diary to monitor the intervention. They are also asked to register hours of sleep during the first and last week of the intervention. AE and COMB groups are also trained to monitor the intensity of their activity using the Borg Rating of Perceived Exertion Scale (BRPES) (Borg, 1982) while CCT and COMB conditions are informed about the computer program. A follow-up calendar is also created to determine adherence and any interfering events. All participants receive phone calls every 2 weeks, a mid-point intervention visit after 6 weeks and a final visit (**Table 2**).

Computerized Cognitive Intervention (CCT)

The intervention program consists of a set of multi-domain cognitive tasks targeting executive function, visual and verbal memory and sustained, divided and selective attention using a computerized telerehabilitation platform called Guttmann NeuroPersonal Trainer® (GNPT®, Spain; Solana et al., 2014;

TABLE 2 | Assessments.

	Enrollment and screening		Baseline assessments (within 2 weeks prior to the start of the intervention)			Intervention			Post-intervention assessments (within 2 weeks after the completion of the intervention)		
	Telephone screening	**Visit 1: Screening**	**Visit 2: Baseline assessment**	**Visit 3: Baseline assessment**	**Visit 4: Baseline assessment**	**Visit 5: Initial intervention**	**Visit 6: Mid-intervention**	**Visit 7: Final intervention**	**Visit 8: Post-intervention assessment**	**Visit 9: Post-intervention assessment**	**Visit 10: Post-intervention assessment**
First review of criteria	X										
Montreal Cognitive Assessment 5-min (MoCA 5-min)	X										
Information of study	X	X									
Exhaustive review of criteria		X									
Informed consent form		X									
Mini-Mental State Examination (MMSE)		X								X	
Geriatric Depression Scale (GDS-15)		X								X	
Medical history			X								
General health status			X						X		
Blood extraction			X						X		
Battery of neuropsychological tests				X						X[1]	
Psychological health and daily activities scales				X						X	
Physical activity questionnaire (VREM)				X						X	
Cardiorespiratory fitness (Rockport 1-mile walk test)				X						X	
Magnetic resonance imaging					X						X
Information about intervention						X					
Follow-up adherence questions[2]							X	X			
Actimeter[3] (Polar Loop®)						X		X			
Intervention follow-up diary[4]						X	X	X			

[1]All of tests except vocabulary subtest; [2]This information is also collected in phone calls every 2 weeks between visits; [3]Participants carry the actimeter the first and last week of training; [4]Participants record the diary every day during the intervention; MoCA 5-min (Wong et al., 2015); MMSE (Blesa et al., 2001); GDS-15 (Martínez et al., 2002); VREM – Reduced Minnesota leisure time physical activity questionnaire (Ruiz et al., 2012); Rockport 1-mile walk test (Kline et al., 1987).

Solana et al., 2015). The cognitive functions trained are assessed with the battery of neuropsychological tests. However, the specific training tasks differ from the task performed in the assessment. GNPT includes a variety of exercises designed by neuropsychologist based on cognitive paradigms. The GNPT® platform calculates an individual profile based on age, educational level and the results of the neuropsychological assessment. The demand of the tasks is adjusted according to the performance of previous activities, which allows the software to design a new activity plan adapted to the participant level in each cognitive domain. Training is done individually at home for 45 min per session. Participants without access to a personal computer or Internet can perform the intervention in the health care center. The software records the numbers of sessions and performance in activities automatically.

Aerobic Exercise (AE)

The training program is based on international guidelines of physical exercise (World Health Organization [WHO], 2010). Participants are instructed to walk briskly in one continuous bout (45 min for 5 days, 225 min per week); intensity and duration are initiated in a stepwise manner in order to reduce the possibility of injury. The first week they must walk 30 min at 9–10 on the BRPES (Borg, 1982) considered light intensity. Time is increased to 45 min with the same intensity during the second week. During the rest of the program (10 weeks), they maintain the 45 min and increase the intensity of the activity to a moderate-high effort that corresponds with 12–14 in the same scale. Subjects are trained to use the BRPES (Borg, 1982) and to record this intensity and frequency of activity in a diary.

Combined Training (COMB)

This group receives both the CCT and the AE intervention. They follow the same previously described instructions for each condition. Participants can organize both tasks at their convenience, always applied in a single continuous bout of 45 min each at any moment of the day. This results in 90 min of daily activity, 5 times per week. We did not set any restriction about the order of the tasks during the day or time-point at which they had to be applied.

Control Group

Participants in the control group are on the waiting list for 3 months and are asked to keep their normal lifestyle. Once the control condition is finished, they have the option to start one of the interventions (CCT, AE, or COMB). Data of this optional activity will not be included in the trial as they are not considered participants during this period.

Safety Considerations

In order to anticipate, prevent and answer medical or personal issues of the participants, several considerations will be taken into account. First, all the assessments are reviewed by the corresponding health professional before randomization to ensure safety during the intervention. Abnormalities identified are reported and these participants are rerouted to the corresponding healthcare service. Participants receive reports of all the assessments. Instructions for each intervention include healthy advice to prevent injuries. Participants can also contact the intervention staff for any problems or pain that they may experience. Adverse events occurring during the intervention are monitored in a diary and sent to a physician in case of medical incident. Participants will be excluded from the trial based on medical recommendation.

DATA MANAGEMENT AND RESULTS

Data Quality

A computerized database is used to collect and organize all data. Data is collected without personal identifying information using a code assigned by the assessor and researchers will only have access to this information in case of an incident. Data from all participants will be collected regardless of whether the participant withdraws from the intervention or not. Assessments, individual reports and databases will be double-checked. We will follow Data Quality Assessment Checklist and Recommended Procedures (DQA; USAID, 2014) that assesses a variety of dimensions as validity, reliability, timeliness, precision and integrity. Regarding interventions parameters, we will assess the coherence between personal diaries, phone-call follow-ups and actimeter. We will analyze if compliance is related to expected physical changes. In addition, if we identify any issues, we will inform and apply any required statistical procedures to control them.

Outcomes

Primary Outcomes

To address the primary hypothesis, an extensive neuropsychological battery was designed by Projecte Moviment. Each test has been selected for its psychometric qualities and high relevance in the area of study. These tests provide measures of multiple functions: executive functions, visuospatial abilities, memory, language, attention and processing speed. We will calculate z-score composites from normalized raw data for each cognitive domain and a global cognitive function score as a sum of all domains (**Table 3**).

Secondary Outcomes

Several domains are assessed to test secondary hypotheses. Main outcomes and measures are described in **Table 4**.

Cognitive decline screening

Montreal Cognitive Assessment 5 min (Wong et al., 2015) and Mini-Mental State Examination (Blesa et al., 2001) assess global cognitive function as relevant markers of cognitive decline.

Psychological health and daily activity

Questionnaires ask for depressive symptoms and emotional status, sleep quality and subjective performance in daily activities. These outcomes will test the potential effect of the interventions to enhance perceived psychological status and functionality which may be related to cognitive effects and other secondary outcomes.

TABLE 3 | Primary outcomes: variables and measures.

Outcome/Variable			Outcome measure
Composites 1st level	**Composites 2nd level**	**Tests – Subtest**	
Executive function	Inhibition	Stroop – Interference	*Z* score
	Working memory	WAIS III – Backward span	*Z* score
		TMT – B	*Z* score
	Fluency	Letter fluency	*Z* score
		Category fluency	*Z* score
Visuospatial function	Visuospatial	ROCF – Copy accuracy	*Z* score
Memory	Verbal memory	RAVLT – Total learning	*Z* score
		RAVLT – Recall II	*Z* score
	Visual memory	ROCF – Memory accuracy	*Z* score
Language	Language	WAIS III – Vocabulary	*Z* score
		BNT (15 items)	*Z* score
Attention – Speed	Attention	WAIS III – Forward span	*Z* score
		WAIS III – Digit symbol coding	*Z* score
		WAIS-III – Symbol search	*Z* score
	Speed	TMT – A	*Z* score
		ROCF – Copy time	*Z* score

Stroop test (Golden, 2001); WAIS-III, Wechsler Adult Intelligence Scale (Wechsler, 2001); TMT, Trail Making Test (Tombaugh, 2004); Verbal fluency tests (Peña-Casanova et al., 2009); ROCF, Rey-Osterrieth Complex Figure (Rey, 2009); RAVLT, Rey Auditory Verbal Learning Test (Schmidt, 1996; Marqués et al., 2013); BNT, Boston Naming Test (Goodglass et al., 2001).

TABLE 4 | Secondary outcomes: variables and measures.

	Variable/Outcome	Outcome measure
General cognitive function	Montreal Cognitive Assessment 5-min (MoCA 5-min)	*Z* score
	Mini-mental State Examination (MMSE)	*Z* score
Psychological health Daily activity	Geriatric Depression Scales (GDS-15)	*Z* score
	Modified version of Visual Analog Mood Scale (VAMS)	*Z* score
	Short Informant Questionnaire on Cognitive Decline in the Elderly (S-IQCODE)	*Z* score
	Pittsburg Sleep Quality Index (PSQI)	*Z* score
	Clinical Outcomes in Routine Evaluation-Outcome Measure (CORE-OM)	*Z* score
Fitness	Cardiorespiratory fitness (Rockport 1-mile walk test)	VO_2 max
Physical activity	Reduced Minnesota leisure time physical activity questionnaire (VREM)	METs
	Actimeter activity parameters (Polar Loop®)	Hours, steps, km, kcal
	Actimeter sleeping parameters (Polar Loop®)	Hours, %
Health status	Weight, height, and waist diameter	Kg, cm
	Blood pressure	mm Hg
	Hypertension, diabetes, and dyslipidemia	Yes/No
	Tobacco and alcohol use	Yes/No, Units
Blood sample data	Hemogram	Conventional units
	Biochemistry in plasma	Conventional units
	Cortisol	ng/mL
	Genetics – Apolipoprotein E (APOE)	E4+/E4-
	Genetics – Brain Derived Neurotrophic Factor (BDNF)	Met+/Met-
	Cytokines	ng/mL
Neuroimaging	T1-weighted	Volume
	T2-weighted turbo inversion	Volume
	Susceptibility weighted imaging	Volume
	Resting state	*Z* score
	Diffusion tensor imaging (DTI)	Fractional Anisotropy Index

MoCA 5-min (Wong et al., 2015); MMSE (Blesa et al., 2001); GDS-15 (Martínez et al., 2002); VAMS (Stern et al., 1997); S-IQCODE (Morales et al., 1992); PSQI (Rico and Fernández, 1997); CORE-OM (Trujillo et al., 2016); Rockport 1-mile walk test (Kline et al., 1987); VREM (Ruiz et al., 2012); MET (Metabolic Equivalent of Task).

Aerobic fitness

CRF is assessed by the Rockport 1 mile walk test. Participants are instructed to walk on a treadmill for 1 mile adjusting their speed in order to be as fast as possible without running (Technogym®, Italy). Maximal aerobic capacity (VO2 max) will be estimated with the linear regression reported by Kline et al. (1987). CRF estimation is a well-known measure of cardiovascular health. We expect to describe the relationship between CRF and the physiological blood measures as well as how change in CRF relate to brain and cognitive outcomes.

Physical activity

Information about energy expenditure of PA performed during the last month is obtained by the Reduced Minnesota leisure time PA questionnaire (Ruiz et al., 2012). Polar Loop® actimeter (Polar Electro, NY, United States) registers daily PA (hours, steps, km, kcal) and sleeping (hours, %) parameters. Energy expenditure is the very first consequence of exercising. We aim to identify if baseline energy expenditure is related to baseline CRF and describe how the change in energy expenditure is related to physiological molecular correlates, CRF and to brain and cognitive outcomes.

Health status

A nurse registers demographic data, blood pressure, anthropometrics measurements, and cardiovascular risk factors. Demographic data will allow us to control the influence of individual variables. We expect that weight loss could be related to the physiological blood markers and to primary outcomes or other secondary outcomes. The reduction of cardiovascular risk factors is an indirect measure of better cardiovascular health that has been related to exercise.

Blood-sample data

Hemogram, biochemical parameters, and lipidic profile will be quantified in a common blood test. Cortisol will be analyzed in plasma and genetic biomarkers in APOE (SNPs rs429358 and rs7412) and BDNF (rs6265) genes will be determined in the buffy coat fraction. Finally, a set of 105 cytokines will be studied semi-quantitatively with The Proteome Profiler Human™ XL Cytokine Array (R&D Systems, MN, United States). Biomarkers with relevant differences within and between groups will be analyzed quantitatively using an ELISA immunoassay method. We have chosen relevant genetic variables and physiological molecular markers that have been related to exercise interventions. We expect to detect changes at 3 months and describe any type of relationship with cognitive outcomes.

Neuroimaging

Magnetic Resonance Imaging will be obtained in a Siemens Magnetom Verio Symo MR B17 (Siemens Healthineers, Erlangen, Germany). The protocol includes: (1) T1-weighted multi-planar reformat sequence (voxel: 0.9 × 0.9 × 0.9 mm, TR/TE: 1900/2.73 ms, slices: 192; thickness: 0.9 mm); (2) T2-weighted turbo spin-echo sequence (voxel: 0.7 × 0.5 × 3 mm, TR/TE: 6000/74 ms, slices: 35, thickness: 3 mm); (3) T2-weighted turbo inversion recovery magnitude (voxel: 1 × 0.8 × 3 mm, TR/TE: 9000/99 ms, slices: 44, thickness: 3 mm); (4) Susceptibility-weighted imaging with T2 – fl3d sequence (TR/TE: 28/20 ms, slices: 88); (5) Resting state imaging with a gradient echo planar imaging sequence (TR/TE: 2000/25 ms, slices: 39, thickness: 3 mm, volumes: 240); (6) Diffusion tensor imaging, echo planar imaging (voxel: 2 × 2 × 2 mm, TR/TE: 10200/89 ms, 64 directions, 1 acquisition). On one side, structural and functional brain outcomes may help identify the molecular changes that are related to changes in brain volume, microstructure and connectivity between groups. On the other, we aim to identify if changes in the MRI outcomes are associated with cognitive changes.

Other parameters from the follow-up such us adherence and type of adverse events will be described.

Analyses

Power Analysis

The sample size was determined considering previous studies (Erickson et al., 2011) and the effect size and the standard error of cardiovascular interventions on all cognitive tasks reported in a previous meta-analytic study (Colcombe and Kramer, 2003). We also considered the following assumptions: bilateral contrasts and an effect size of 0.4 using the Tukey–Kramer multiple comparison test at 0.05 significance level with a common standard deviation within a group of 0.3. In order to have at least 80% statistical power to answer the 3 primary aims of the project, we need 18 subjects in the control group and 36 for CCT, AE, and COMB groups, respectively. Assuming 10% of lost to follow up (Rahe et al., 2015), we need a total sample size of 139 subjects (control group n = 19, intervention groups n = 40). Sample power has been computed using PASS 14 Power Analysis and Sample Size Software.

Statistical Analyses

Addressing the primary objective, statistical procedures will be performed with IBM SPSS Statistics 24 and R Environment. The distribution of raw scores will be examined in order to assess data quality (i.e., outliers, skewness) and we will obtain sample z-scores for all cognitive tests. Five primary domains will be calculated by adding z-scores – executive function, memory, language, attention-speed, and visuospatial function – which will be split into nine secondary domains in order to assess specific changes within each domain – inhibition, fluency, working memory, verbal memory, visual memory, language, attention, speed and visuospatial function (see **Table 3**). Domains will be based on the literature (Strauss and Spreen, 1998; Lezak et al., 2012) given the fact that a principle component analysis would not be appropriate for our sample size.

In order to test our primary hypotheses, we will conduct an intention-to-treat (ITT) analysis considering data from all randomized participants, including those that complete and drop-out, in order to prevent attrition bias. An adequate method of imputation will be applied and informed. Parametric or non-parametric tests will be chosen regarding the fitting of data to statistical requirements of the tests and we will follow a coherent pipeline to explore variance: (a) comparison of baseline values between groups to identify potential variables to adjust further analyses; (b) comparison of variables at different

time-points for each group to identify the independent effect of each condition; (c) identification of significant cross-time correlations in order to determine whether it is necessary to control for baseline measures; (d) interaction between conditions and time-points to compare interventions. Sex, age, and years of education will be considered covariates beforehand and a two-tailed p-value < 0.05 will be set as the significant threshold and the corresponding correction for multiple comparisons will be applied. In a second phase, we will define a per-protocol (PP) sample including only subjects that finished the intervention with at least 80% adherence and we will reproduce the same pipeline. In case of high disparity between ITT and PP results, we will analyze potential variables related to that discrepancy.

We will analyze our secondary hypotheses following the same pipeline in the per-protocol sample to guarantee that we will be studying the effects of the intervention on the previously described outcomes. We will examine potential mediator effects through relationships between primary and secondary outcomes accounting for the intervention condition using adequate correlational methods and linear mixed models. Structural neuroimaging data will be first processed studying whole brain, hippocampus and frontal lobe volumes and white matter microstructure. In a second phase, connectivity and white matter lesions will be analyzed. Data will be published in several papers where detailed procedures and software packages will be described.

DISCUSSION

Healthy aging is a current social challenge. Lifestyle behaviors such as cognitive training and exercise have a positive impact on health, brain and cognition with the possibility of greater benefits when they are combined. Despite this evidence, there are still many questions remaining unanswered. Questions about the type of activity, length, frequency, duration, and intensity required to observe a cognitive effect, the potential individual predictors of response to the intervention, the relationship between physiological molecular correlates, and structural and functional brain changes and cognitive and psychological benefits remain unclear. Projecte Moviment is a multi-domain intervention trial based on prior evidence that aims to understand the effects of CCT, AE, and COMB on cognitive and psychological outcomes compared to a passive control group and determine related biological correlates as well as significant predictors of their effect. We aim to describe what type of change these specific interventions may produce on biological, cognitive and psychological outcomes at 3 months. These results may support the literature that is currently examining the timeline of the effects of these interventions (Stimpson et al., 2018; Cabral et al., 2019) on cognition and trying to identify potential related modifiers (Barha et al., 2017; Northey et al., 2017). We expect to find changes in physiological molecular correlates as well as structural and functional brain outcomes within each intervention group and determine how they differ across groups. Those changes may be related to potential cognitive and psychological improvements depending on adherence, characteristics of the intervention and other individual variables.

Projecte Moviment aims to overcome some of the limitations underlined in relevant reviews (Daskalopoulou et al., 2017; Carrion et al., 2018). First, we examine several cognitive domains and multiple dimensions of health collecting information at different levels of measure. This fact will allow us to examine the effect on different cognitive domains. We will be able to identify other related variables that may explain the results and differences between groups at a molecular level. To our knowledge, it is one of the first trials to propose a high-frequency program, 5 days per week for 3 months in an ecological environment. We chose a short period of time, used in other trials (Pereira et al., 2007; Renaud et al., 2010; Maass et al., 2015; Cabral et al., 2019), but with a higher frequency to examine if we can observe the same or greater biological changes and equivalent or greater related cognitive improvements. A home-based non-reimbursed participation may help us to determine if adherence patterns and effects are like center-based rewarded interventions which might be helpful for clinical guidelines. We will also control the influence of many demographic variables through the eligibility criteria of the sample and age and sex balanced groups.

Nevertheless, we are aware of the limitations of the current study. Highly demanding home-based interventions during a short period of time may result in low adherence and an insufficient amount to test our hypotheses about effects on cognition. Intention to treat and per protocol analyses will help us to describe discrepancies and control attendance. We are also collecting data at a molecular, structural and behavioral level in order to identify the effect of the intervention at multiple biological and behavioral levels. Despite the short duration, Stimpson et al. (2018) proposed a timeline of the effects of exercise intervention with changes in the blood and brain parameters within 3 months. In addition, literature suggested that middle-age adults and healthy participants may lead to null results (Erickson et al., 2014; Young et al., 2015). We believe that replication and deeper examination and understanding of discrepancies is needed. These and future limitations, will be considered when analyzing, interpreting and publishing all results.

Projecte Moviment aims to report results through at least 6 publications in peer-reviewed journals without restrictions to positive or negative results. Conclusions will also be presented in oral communications and posters at national and international conferences. We will inform participants and the general community through educative releases. Projecte Moviment aims to reach health professionals to support the translation of the results of the current study into clinical practice.

Future research will also include a large study of gene expression and metabolites in this sample following big data analytic strategies under the concept of omics to provide a deeper understanding of the biological mechanisms related to these interventions.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of SPIRIT Guidelines with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Bioethics Commission of the University of Barcelona (IRB00003099) and Clinical Research Ethics Committee of IDIAP Jordi Gol (P16/181).

AUTHOR CONTRIBUTIONS

MM conceptualized the study and contributed to the study design and implementation as Principal Investigator. PT-M and KE made substantial contributions to the design and content of the trial. MV, MTA, RF, GP, RD-A, JS-R, CC, AP, GG, JM, and SD contributed to the design of the trial from their area of expertise. JT, AG-M, MH-P, and MTA collaborated in the implementation of specific procedures. AC-S, FR-C, and NL-V contributed to the design, implementation, and writing of the protocol. All authors reviewed the manuscript and provided the final approval for the manuscript.

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MINI REVIEW
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In the Long Run: Physical Activity in Early Life and Cognitive Aging

*Charlotte Greene[1], Hyunah Lee[2] and Sandrine Thuret[2]**

[1] GKT School of Medical Education, King's College London, London, United Kingdom, [2] Department of Basic and Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, United Kingdom

A certain degree of age-related cognitive decline is normal; however, some people retain more cognitive function than others. Cognitive impairment is associated with an increased risk of dementia. Thus, understanding the factors that contribute to cognitive reserve is crucial, so effective strategies for the prevention of dementia can be developed. Engaging in physical activity can delay cognitive decline and reduce the risk of dementia and a number of early life conditions have been shown to have long-lasting effects on cognition. This mini-review combines these two observations to evaluate the evidence from both animal models and epidemiological studies for physical activity in early life (≤30 years) delaying cognitive decline in later life (cognition tested ≥60 years). Three epidemiological studies were found; two showed a positive association and one found none. The latter was deemed to have an unreliable method. A review of animal studies found none that analyzed the effect of physical activity in early life on cognition in later life. However, in rodent models that analyzed mid-life cognition, runners showed improved cognition and enhanced adult hippocampal neurogenesis, changes which were preserved across the life span. Currently, there is insufficient evidence to conclude whether physical activity in early life may delay cognitive decline in later life, but these results indicate that further studies are warranted. Future human research should be in the form of longitudinal studies that begin below ≤15 years and assess sex differences. Crucially, the physical activity data must define type, quantity and intensity of exercise.

Keywords: cognition, cognitive decline, exercise, early life, old age, cognitive reserve, prevention

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Luiz Royes, Universidade Federal de Santa Maria, Brazil

***Correspondence:**
Sandrine Thuret
sandrine.1.thuret@kcl.ac.uk

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INTRODUCTION

The aging brain exhibits widespread detrimental changes in structure, including reduction in size, plasticity and cerebral blood flow, and also in function (Peters, 2006). While some age-related cognitive decline in memory (particularly semantic memory), executive function, and processing speed may be considered normal (Harada et al., 2013), more significant decline in these than expected for a person's age can be classified as either cognitive impairment with no dementia (CIND) or mild cognitive impairment (MCI), depending on which cognitive domains are most affected (Blondell et al., 2014). A diagnosis of CIND or MCI has been associated with increased risk of dementia (Blondell et al., 2014). A large-scale, longitudinal study of individuals aged ≥55, showed that those with MCI were 3.89 times more likely to be diagnosed with dementia later in life than those without (de Bruijn et al., 2014).

Dementia is a devastating condition not only for the individual and their loved ones, but also poses an enormous and growing social and economic burden for public health. Currently, over 5% of the aged ≥65 population suffer from dementia and by 2050 it has been estimated that 22% of the entire population will be aged ≥60, equating to approximately two billion people of that age group (Blondell et al., 2014; Shatenstein et al., 2015). To reduce the incidence of dementia, delay the age of onset, and improve quality of life for this subpopulation, we need to understand what factors can contribute to slowing age-related cognitive decline.

Evidence suggests engaging in physical activity may delay cognitive decline and reduce the risk of dementia and a number of early life conditions have been shown to have long-lasting effects on cognition. In this review, we combine these two observations and evaluate the existing evidence from epidemiological studies for physical activity in early life (≤30 years) delaying cognitive decline in later life (≥60 years) and from rat models on the effect of physical activity in early life (≤4 months) on cognition in later life (≥7 months). First, we explore the idea of cognitive reserve, and the significance of early life events on its development. Next, we discuss why physical activity is an important factor to consider. We then review the data generated from epidemiological studies and animal models that met our inclusion criteria and search terms.

EARLY LIFE CONDITIONS AND COGNITIVE RESERVE

The brain is capable of change and adaptation as a result of experience, termed plasticity. Previous studies have suggested there is no direct relationship between brain pathology and cognitive impairment (Walhovd et al., 2016). Alzheimer's disease lesions are commonly found upon post-mortem in people who were cognitively intact before death, termed asymptomatic AD (Iacono et al., 2009). Likewise, individuals with similar levels of brain pathology can show different levels of cognitive impairment (Fritsch et al., 2007). These observations have led to the rise of the term "cognitive reserve" – the idea that people can build up a reserve of neurological and/or cognitive resources that increase the tolerance toward damaging stimuli. Various studies have shown factors that might serve as the biological basis of cognitive reserve including greater cortical surface area and neuronal hypertrophy (Iacono et al., 2009; Walhovd et al., 2016). Hypertrophy of the cell bodies (+44.9%), nuclei (+59.7%), and nucleoli (+80.2%) of neurons in the CA1 region of the hippocampus was found in the brains of those with asymptomatic AD compared with those with MCI, suggesting that this may be one of the mechanisms that protects against cognitive decline (Iacono et al., 2009).

Recently, there has been increasing interest into whether early life conditions can affect this reserve. Lower birth weight, body length and head circumference, have been shown to correlate with faster cognitive decline and increased incidence of dementia rates (Raikkonen et al., 2013). Childhood nutritional status (Zhang et al., 2010), linguistic ability (Calvo et al., 2016), stress and adversity (Radford et al., 2017), and education (Zahodne et al., 2015) have also been linked to cognition in later life.

PHYSICAL ACTIVITY AND COGNITIVE FUNCTION

Evidence from both epidemiological studies and randomized control trials suggests that engaging in physical activity improves cognitive function at various stages of life, from early (Sibley and Etnier, 2003; Scudder et al., 2014) to mid and late life (Yaffe et al., 2001; Jedrziewski et al., 2010; Nishiguchi et al., 2015). In a meta-analysis of 47 longitudinal studies with participants aged ≥40 years, Blondell et al. (2014) showed that people with higher levels of physical activity had a lower risk of progressive cognitive decline (RR 0.65, 95% CI 0.55–0.76) and dementia (RR 0.86, 95% CI 0.76–0.97).

Contradictory evidence was presented by Sabia et al. (2017) who found no neuroprotective effects of mid-life physical activity and suggested that previous findings were attributable to reverse-causation (people in the pre-clinical dementia phase exercise less). Their follow-up time of 28 years is the longest of the studies discussed. However, the study with the longest follow-up time, 26 years, in the review by Blondell et al. (2014) found a significant association between physical activity and cognition. The longer-term impact of physical activity across the life-span merits more attention. Indeed, in their sample of 226 middle aged and older adults (≥55 years), Gill et al. (2015) observed that better global cognitive performance was associated with higher lifetime physical activity ($p = 0.045$), physical activity between 0–20 years ($p = 0.036$) and 21–35 years ($p < 0.0001$).

Numerous animal studies have shown positive correlations between exercise and cognition and proposed several possible neurobiological mechanisms for the enhancement of cognitive reserve – adult hippocampal neurogenesis and suppressed apoptosis, increased production of insulin-like growth factor and brain-derived neurotrophic factor (BDNF) and modulated cytokine signaling (Kim et al., 2010; Cassilhas et al., 2012; Gomes da Silva et al., 2012; Speisman et al., 2013). Enhanced neurogenesis in the dentate gyrus (DG) of the hippocampus has received particular attention. Neurogenesis in the mammalian DG declines with age, while an increase leads to improved cognitive function (Kempermann, 2015). Exercise in rodents can induce a 2–3 times increase of newly generated hippocampal neurons, an effect detected as early as 24 h on, and improve neuronal complexity by boosting dendritic spine density (Saraulli et al., 2017).

Research into whether different levels of exercise intensity produce varying effects on cognition has yielded inconsistent results. In rats, running at a speed of 25 m/min for 12 weeks (the severe exercise group) led to shrunken hippocampal neurons with damaged mitochondria (Sumitani et al., 2002) and only low-intensity running (15 m/min), not moderate (25 m/min), increased the expression of BDNF in the hippocampus (Soya et al., 2007). Indeed, at moderate-intensity running, induction of BDNF mRNA was depressed

(Soya et al., 2007). Inoue et al. (2015a,b) more precisely defined the intensity of exercise by training rats below (aerobic) and above (anaerobic) the lactate threshold, discovering that adult hippocampal neurogenesis was increased only by aerobic exercise. However, in their study Lee et al. (2018) found that both intense and moderate exercise prevented cognitive decline from chronic stress and improved newborn cell survival and blood vessel density. Moderate exercise sessions involved running at 20 m/min for 60 min, while during intense sessions the treadmill speed was increased from 30 m/min until the mouse became exhausted (most at 50 m/min). The blood lactate concentration exceeded 14 mmol/L immediately after running, suggesting that the lactate threshold was exceeded.

The results of human studies are also mixed. One study reported that individuals who received aerobic training showed substantial improvements in cognitive function compared to those on the anaerobic program (Kramer et al., 1999). In a study of 36 female college students the high-intensity resistance (primarily anaerobic) group (resistance exercises; 28 repetitions, 80% 1RM) showed a worse cognitive performance compared to those on a moderate-intensity mixed program (resistance exercises; 12 repetitions, 30% 1RM, aerobic; 30 min walking) and controls (Chang et al., 2017). While, a meta-analysis of 17 studies found that both aerobic and resistance exercises were effective in improving cognition in adults with MCI (Wang et al., 2019).

Brown et al. (2012) measured routine levels of physical activity via actigraphy in 217 participants aged 60–89 years and reported a significant association between intensity, but not volume, of physical activity and cognitive functioning. Analysis was done by stratifying the cohort into tertiles based on physical activity intensity. Given this method and the age of participants it seems unlikely that those in the highest tertile of physical activity intensity reached the intensity of the training of other studies (e.g., Chang et al., 2017). The conflicting results in different studies may well be partially explained by the difficulty in defining "high intensity" and the heterogeneity of classifications used. The question of what level of intensity, and type (aerobic or resistance/anaerobic) may be most beneficial for cognitive function and enhancing cognitive reserve needs to be researched further.

EPIDEMIOLOGICAL STUDIES ON THE EFFECTS OF EARLY PHYSICAL ACTIVITY ON LATE LIFE COGNITION

Children's brains are uniquely malleable, but the brain continues to develop into the third decade of life (Johnson et al., 2009; Petanjek et al., 2011). Thus, early life will be defined as ≤30 years. An adequate assessment of cognitive aging is defined as cognitive tests completed ≥60 years, when structural age-related changes to the brain would have begun (Nyberg et al., 2012). A further inclusion criterion was the use of statistical adjustment, including adjusting for exercise at other times in life, to ensure it was specifically exercise in the early period that was analyzed. Exclusion criteria were not in English, a conference paper and published later than 2018. The following search terms – ((((early life OR adolescence OR early adulthood)) AND (physical activity OR exercise)) AND (cognitive aging OR cognit*)) AND (old age OR late) – were applied on PubMed and Embase. Three studies met the inclusion criteria.

As shown in **Table 1**, two studies showed a positive association between early life exercise and cognitive function later in life (Dik et al., 2003; Middleton et al., 2010), and one study showed no association (Fritsch et al., 2007). None looked at the effect of exercising ≤15 years of age, an obvious immediate gap in research.

Fritsch et al. (2007) drew participants from the population of a longitudinal aging study of students who graduated from high school 1945–1947. The authors assessed the extent of students' physical exercise while attending the school aged 16–18 by gathering information from their yearbooks. They defined physical activities as "athletic clubs including sports teams, dance clubs, honor groups associated with athletic performance, and cheerleading" and grouped the participants based on the number of activities mentioned in the yearbook. Although this data is free from recall error and bias it has significant weaknesses; it covers the short period of 2 years and only exercise in school. Most importantly, the analysis is based on the number of activities rather than the time spent engaged in them. Someone who excels at one sport may well be a member of only that team. They could devote significant time to it, but they would be considered by this study to have a low level of physical activity. This method also does not include analysis of the intensity of physical activity, an important omission given the evidence discussed above that it may be specifically mild, aerobic forms that are beneficial. Thus, the conclusion of no observable association is unconvincing and can be ignored unless the results are replicated in other independent studies.

The two studies that showed a positive association had substantially larger sample sizes (1241 and 9334 vs. 349). Dik et al. (2003) found an improvement in processing speed, but not global/general cognitive functioning (GCF), only in men in the low (≤1–2 h per week) and moderate exercise (3–9 h per week) groups. The authors provide a convincing explanation for why this trend was not seen in the high-level exercise cohort (≥10 h per week). Fifty-six percent of those men had a high level of physical work. Not only may manual labor be associated with poor working conditions and exposure to harmful substances, it is likely to be a static and anaerobic form of exercise. Indeed, separate analysis for sport and work-related activities revealed the negative association of the high exercise men could be attributed to physical work. In contrast, Middleton et al. (2010) observed a positive association in 9344 women. Teenage physical activity was correlated with lower odds of late-life cognitive impairment (defined as an mMMSE 1.5 SD ≤ mean) compared to physical inactivity – 8.5 vs. 16.5%. Four age categories were included in the study (teenage, 30, 50, and late life) and when a separate comparative analysis was

TABLE 1 | Characteristics and results of studies investigating the correlation between early life (≤30) physical exercise and cognitive aging (≥60).

Study/country	Participants	Physical activity	Cognitive tests	Statistical adjustment	Results after full adjustment
Dik et al., 2003/Netherlands	1,241 – M (48.7%) and F (51.3%), aged 62–85 (mean 74.9), CI excluded, No info on ethnicity	15–25 years Asked retrospectively no. of hours per week	GCF (MMSE), SP (Alphabet coding task-15)	Confounders: age, sex, verbal intelligence, SES, lifestyle (early life physical work, current physical activity, smoking, alcohol), health indicators (diabetes, cardiac disease, depression)	GCF – no significance, SP – M only positive association for low (beta = 0.97) and mod (0.67) high – insignificant negative association (−1.04)
Fritsch et al., 2007/United States	349 – M (42.4%) and F (57.6%), aged 74 – 76 (mean 74.8), CI excluded, 99.7% white	16–18 years Information collected from yearbooks – grouped according to number of physical activities	GCF (TICS-m), SP (Timed months of the year backward test), episodic memory (Logical memory A subtest of the wechsler memory scale), verbal fluency (animal naming)	Path analysis for: sex, teen IQ, parent's SES, HS physical, mental, social activities, ML mental, physical and social occupational demands, education	No association
Middleton et al., 2010/United States	9344 – only F, ≥65 years (mean 71.6), "primarily white" – no figures provided	"teenage" Asked retrospectively about low, mod, high intensity exercise – modified Paffenbarger questionnaire	GCF (mMMSE)	Confounders: age, education, marital status, diabetes, hypertension, depressive symptoms, smoking, BMI	Physically active lower prevalence of CI vs. inactive – 8.5 vs. 16.7% 0.65 OR (0.53–0.80)

M, men; F, female; CI, individuals with cognitive impairment; GCF, global/general cognitive functioning; SP, speed processing; HS, high school; ML, midlife.

carried out teenage physical activity was most strongly associated, OR = 0.73 (0.58–0.92).

In their assessment of GCF all three studies used a version of the Mini-Mental State Examination (MMSE; Folstein et al., 1975). Dik et al. (2003) used the MMSE itself, Middleton et al. (2010) the modified MMSE (mMMSE), and Fritsch et al. (2007) the Modified Telephone Interview for Cognitive Status (TICSm; Welsh et al., 1993). The mMMSE is a shortened version which evaluates orientation, concentration, memory, and praxis but omits questions regarding language. The TICSm was developed as a telephone version of the MMSE and correlates highly with it (r = 0.8) (Fritsch et al., 2007). It includes orientation, concentration, memory, naming, comprehension, and abstraction. The MMSE is a widely used and respected tool designed as a screening test for cognitive impairment with a maximum score of 30, and normal cognitive function usually set at a score ≥24 (Creavin et al., 2016). A recent Cochrane review (Creavin et al., 2016) with the objective of determining the diagnostic accuracy of the MMSE for dementia in patients ≥65 found that the summary accuracy at a cut point of 24 was sensitivity 0.85 and specificity 0.90. For speed processing Dik et al. (2003) employed the Alphabet Coding Task-15 (Piccinin and Rabbitt, 1999) while Fritsch et al. (2007) used the Timed Months of the Year Backward Test (Ball et al., 1999), both are validated timed tests for the assessment of central speed processing. The fact different cognitive tests were used is a limitation when comparing the results of the studies. However, there are only minor differences between the assessments used and the MMSE as an effective measure of cognition is supported by a strong evidence base.

A limitation of two of the three studies is that the data provided about exercise is retrospective and self-reported by participants. Asking people, some of whom may have cognitive impairment, to report on their exercise levels of decades before, can be problematic, making defining the quantity, intensity and type of exercise difficult. A shared weakness of all three is that the majority of participants were white. Most importantly, isolating the impact of physical exercise in early life given all the confounding variables throughout life is challenging.

Given only three studies could be found that fit our inclusion criteria, there is insufficient evidence to draw a conclusion. However, the results suggest that further research is warranted. Animal models can remove confounding variables more effectively than human studies and may also provide evidence of a mechanism.

ANIMAL STUDIES ON THE EFFECTS OF EARLY PHYSICAL ACTIVITY ON LATE LIFE COGNITION

The following inclusion criteria were used for rat models; a period of exercise ≤4 months of age, no further exercise after this, and neurogenesis or cognition tested ≥7 months of age. Exclusion criteria were not in English, a conference paper and published after 2018. In an initial search no animal models were found that analyze the effect of physical activity in early life on cognition in later life and thus the age limit was reduced to 7 months (corresponding to mid-life). The impact on mid-life cognition is relevant and worthy

of discussion. Evidence of a neurobiological mechanism that can explain how exercise may exert a long-term effect on cognition, supports the idea that there should be future research into this subject.

Our criteria and the following search terms – ((((early life or early age)) AND (running or exercise)) AND (neurogenesis OR learning or cognt*)) AND (adult or aging or old age) – were applied on PubMed and Embase and yielded two results.

Shevtsova et al. (2017) divided one-month-old male rats into two groups, control (n = 40) and runners (n = 40) that had free access to a running wheel for 6 weeks. After 4 months, the rats were trained on a contextual fear (CF) conditioning task, the learning of which is known to depend on newborn hippocampal neurons. Two weeks later (aged 7 months) they tested for memory of the CF response in the same, similar or different environment. Runners performed better and froze less in similar and different conditions than non-runners, showing that their memory was enhanced. Interestingly, all rats learned the CF task at the same rate. It was specifically the runners' memory that improved (Shevtsova et al., 2017).

The study's second objective was to compare neuronal activity in adult-born and developmentally born neurons in the DG. Three weeks before CF conditioning, rats were injected with chloro-deoxyuridine (CldU), a thymidine analog, to mark a proportion of 5-week-old neurons at the time of testing. To see the activity of these neurons, an immediate early gene (c-Fos) was labeled, and the densities of double-labeled cells (c-Fos/CldU) were measured. The results were that these adult-born neurons were more active than developmentally born neurons, with a higher percentage active in early runners compared to controls. This is evidence for two significant points; that adult neurogenesis is important for learning and memory and that it is enhanced in the long term by physical exercise early in life.

Merkley et al. (2014) divided 4-week-old male rats into controls (n = 24) and runners (n = 28) that had access to a running wheel for 30 days. Both cohorts were subdivided into four groups based on the time between cessation of running and perfusion. They were perfused 1 week, 5 weeks, 6 months, and 9 months after the removal of the wheel. Various immunohistochemistry labels were used to mark stages of neuronal development in the DG. The results showed significant age-related declines in the number of all types of cells in both cohorts indicating that neurogenesis decreases with age. 5 weeks post-running rats (14-week-old) had significantly more adult-born neurons (4-week-old) that had survived and matured than controls (2300 vs. 1500 mean number of cells per DG). The rate of decay of these neurons with age was almost identical in both cohorts. Therefore, exercise-induced changes were preserved across the life span. This is further evidence that early life exercise has long term effects on DG neurodevelopment.

A limitation of both studies is that only male rats were tested. This is particularly relevant given the sex-dependent trends in the epidemiological data and the results of a rodent study that found running enhanced neurogenesis solely in males (Saraulli et al., 2017).

Rats were tested at 7–11 months of age, which as discussed above corresponds to mid-life rather than late-life. Only after further studies have tested later in life (e.g., ≥15 months when reproductive senescence begins) can findings be considered comparable to the effect of physical activity in early life on age-related cognitive decline in humans. The results of these two studies of mid-life cognition suggest that such models should be carried out.

CONCLUSION

The paucity in both human and animal studies is clear. Of the three epidemiological studies that analyzed early life exercise ≤30 years and cognitive function ≥60 years, two found a positive association and one found none. While the latter (Fritsch et al., 2007) seems to have used a relatively unreliable method for assessing the amount of physical activity, the two larger-scale studies did find a positive association. However, Dik et al. (2003) observed a positive association only in processing speed, not in GCF, and only in men in the low and moderate exercise groups, whereas Middleton et al. (2010) found association in their cohort of women and for GCF.

A review of animal studies found none that analyzed the effect of physical activity in early life on cognition in later life. However, in rat models that analyzed mid-life (≥7 months) cognition, runners showed improved cognition and enhanced adult hippocampal neurogenesis, changes which were preserved across the life span. This provides a possible neurobiological mechanism for how early life exercise may exert long term effects on cognition.

There is insufficient evidence to conclude physical activity in early life delays cognitive decline in later life. However, the results from both epidemiological and animal studies indicate that further studies are warranted. Future human research should be in the form of longitudinal studies that address the lack of data on physical activity ≤15 years and the conflicting results for men and women by performing sex stratified analysis. Crucially, the physical activity data must define type, quantity and intensity of exercise. The results of Dik et al. (2003) and a significant body of evidence discussed above suggests that the benefit of physical activity may vary with intensity and type (aerobic or anaerobic/resistance).

AUTHOR CONTRIBUTIONS

CG carried out the study and wrote the manuscript. HL reviewed and contributed to the writing of the manuscript. ST conceived and reviewed the manuscript.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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The Beneficial Effect of Physical Exercise on Cognitive Function in a Non-dementia Aging Chinese Population

Sun Lin[1†], Yang Yang[2†], Qiu Qi[1], Li Wei[1], Nie Jing[1], Zhang Jie[3], Li Xia[1] and Xiao Shifu[1*]*

[1] Alzheimer's Disease and Related Disorders Center, Department of Geriatric Psychiatry, Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Shanghai I-Zhaohu Senior Care Services Co., Ltd., Shanghai, China, [3] Key Laboratory of Arrhythmias, Ministry of Education, Shanghai East Hospital, Tong Ji University School of Medicine, Shanghai, China

Numerous observational studies have shown that physical exercise promotes cognition in the elderly, however, the results from randomized clinical trials (RCTs) are ambiguous. In addition, potential benefits of exercise in an elderly Chinese population have not been comprehensively addressed. In this study, an investigation was launched which focused on the relationship between physical exercise and cognitive function, blood lipid profiles and brain anatomy in a non-dementia aging Chinese population. A total of 2074 non-dementia elderly subjects were included (self-selected exercise n = 1372; self-selected non-exercise n = 702). Amongst the subjects, 689 volunteered to receive blood lipid tests, 141 undergo brain magnetic resonance imaging (MRI), and 1399 receive a 1 year cognitive evaluation follow-up. The Beijing version of the Montreal Cognitive Assessment (MoCA) and the Mini-Mental States Examination (MMSE) were used to assess cognitive function. A significant difference in cognitive function was observed at the baseline and during the 1-year follow-up between the self-selected exercise and self-selected non-exercise groups, however, no significant differences in blood lipids and brain anatomy was evident. Physical exercise has a beneficial effect on cognition, particularly visuospatial function, and decreases the risk of dementia in a Chinese aging cohort.

Keywords: exercise, cognitive function, dementia, brain anatomy, lipid

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Qianhua Zhao, Fudan University, China
Rong Xiao, Capital Medical University, China

***Correspondence:**
Li Xia
ja_1023@hotmail.com
Xiao Shifu
xiaoshifu@msn.com

[†] These authors have contributed equally to this work

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BACKGROUND

Aging is an irreversible process and the number of elderly is rapidly increasing. One quarter of the global population will be at least 65 years old in 2020 (Bherer, 2015). Although the occurrence of disability in the elderly is steadily decreasing, the ever-growing population will likely experience a dramatic increase in the prevalence of cognitive dysfunction disorders (Manton et al., 2006).

Abbreviations: ACSM, American College of Sports Medicine; CHOL, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; MMSE, Mini-Mental States Examination; MoCA, Montreal Cognitive Assessment; RCTs, randomized clinical trials; TG, triglyceride.

In 2015, approximately 47 million people worldwide were living with dementia. This number is projected to reach 115.4 million by 2050 (Prince et al., 2013), emphasizing the need for a preventative health strategy.

Lifestyle contributes to cognitive function and around 35% of the occurrences of dementia in the elderly are attributable to controllable risk factors including physical inactivity, hypertension, obesity, diabetes, and et al. (Livingston et al., 2017). In this regard, accumulating evidence suggests that exercise has a profound effect on brain plasticity and cognitive function. However, a number of observational studies identified that a negative relationship exists between exercise and the risk of dementia (Livingston et al., 2017). A single meta-analysis of 15 prospective cohort studies involving 33816 non-dementia individuals for 1–12 years follow-up reported that physical activity significantly benefits and maintains cognitive health (Sofi et al., 2011). Furthermore, previous publications had found that exercise and fitness had positive effects on the volume of the hippocampus and CHOL levels (Erickson et al., 2011). Despite these findings, a number of RCTs reported that exercise does not benefit mild cognitive impairment when compared to no interventions or cognitive training in elderly subjects (Legault et al., 2011; Barnes et al., 2013; Sink et al., 2015). Studies conducted in several countries reported the relationship between physical exercise and cognitive or cerebral anatomical changes, but few multi-center studies on non-dementia elderly subjects have been performed on the Chinese population. A 5 years follow-up involving 454 elderly Chinese adults without dementia suggested that prolonged exercise produced positive effects on cognition (Ma et al., 2017). However, more researches focused on cognitive impairment or dementia elderly (Lam et al., 2011; Lam et al., 2012; Ho et al., 2015). Here, in this study, we explored the relationship of physical activity with cognitive function, lipid profiles and brain anatomy in a cohort of non-dementia elderly Chinese individuals. We hypothesized that self-selected exercise (self-reported exercise history) associated with higher cognitive function, lower lipid levels, and higher volumes.

SUBJECTS AND METHODS

Subjects

This was a cross-sectional investigation supported by the National Pillar Program of the China Ministry of Science and Technology (project number: 2009BAI77B03). The study was performed across a range of cities including Shanghai, Beijing, Hefei, Nanchang, Ningbo, Xi'an, and Hangzhou from 2011 to 2012 (Xiao et al., 2013). All subjects were required to meet the following criteria for inclusion in the study: (1) Han Chinese, ≥55 years old; (2) absence of dementia; (3) in accordance with the MiniMental State Examination (MMSE) (Folstein et al., 1975) cutoff score, uneducated subjects ≥18, elementary school educated subjects ≥21, and higher than middle-school educated subjects ≥25; (4) no major medical abnormalities, including nervous system disease or unstable, acute or life-threatening medical ailments; and (5) able to complete the study. A total of 2074 elderly subjects without dementia were included in this study (self-selected exercise n = 1372; self-selected non-exercise n = 702). Elderly underwent a screening process that included medical history, physical and neurological examinations, and cognitive assessments. All subjects were assessed by clinical physicians to diagnose whether dementia or not through face-to-face interviews. All assessors accepted the consistency training about cognitive function assessments. Life styles including drinking, smoking, tea, and physical diseases including sleep disorder, hypertension, diabetes were all recorded. Out of the subjects, 1399 individuals were willing to be re-assessed after 1 year (self-selected exercise n = 915; self-selected non-exercise n = 484), 689 individuals accepted baseline blood tests (self-selected exercise n = 411; self-selected non-exercise n = 278), 141 individuals accepted brain MR imaging (self-selected exercisers n = 82; self-selected non-exercisers n = 59). Individuals with a history of mental disease or other disorders that could affect cognitive function were excluded. The Beijing version of the MoCA (Nasreddine et al., 2005) and MMSE (Folstein et al., 1975) were used to measure cognitive function. These screening tests consisted of 30 items that measured multiple cognitive domains (including visual space, memory, naming, attention, calculation, abstract, orientation, and language function). The MoCA test contained more attention-executive items than the MMSE. MoCA was sensitive to detect mid cognitive impairment, and MMSE was suited to distinguish dementia.

The subjects were divided into two groups based on exercise history. The definition of exercise in this study referred to ACSM (Garber et al., 2011). We involved self-selected exercisers that met the following criteria. (1) Time: ≥20 min/day; (2) Intensity: moderate intensity (i.e., brisk walking, jogging, climbing stairs, *etc.*) and/or vigorous intensity (i.e., long-distance running, rope skipping, basketball, *etc.*); (3) Frequency ≥4 days/week. The self-selected exercise group was subdivided into two groups based on the cumulative period of: (1) ≥ 10 years and (2) 1–9 years. We involved self-selected non-exercisers that didn't met the above criteria, including (1) Time: <20 min/day or Intensity: lower than moderate intensity; (2) Frequency <1 day/week. Individuals with uncertain exercise conditions were excluded. Prior to the study, all subjects signed consent forms. Ethical approval was obtained from the Ethics Committee of the Shanghai Mental Health Center.

Measurement of Blood Indexes

Peripheral blood samples were collected from 7 to 9 a.m. Following an overnight fasting period (≥12 h fasting duration). Clot activating gel-containing serum separator tubes and anticoagulant tubes were used to assay blood indexes. Lipid profile analysis including CHOL, LDL, HDL and triglyceride were measured in Shanghai Mental Health Center.

MR Image Acquisition and Processing

MR images were acquired using a Siemens Magnetom Verio 3.0T scanner (Siemens, Munich, Germany). T_1-weighted images were obtained from 176 sagittal slices using 3D magnetization prepared rapid gradient echo acquisition sequence with the following parameters: TR = 2300 ms, TE = 2.98 ms, Flip angle = 9°, spatial resolution = 1*1*1.2 mm^3.

T_1-weighted images were processed into surface-based structural data using the automated reconstruction function in the downloaded FreeSurfer version 6.0 software[1] described by Dale et al. (1999). FreeSurfer was applied to segment brain gray matter, white matter and cerebrospinal fluid, and reconstruct the brain white-gray matter boundary surface. Measurements of cortical thickness, cortical volume, and hippocampus volume for each individual was extracted directly using FreeSurfer.

Data Analysis

Demographics, lifestyle and physical disease were analyzed using a general linear model test for continuous variables and a χ^2 test for categorical variables between the different groups. The distinguishing factors between two groups signed with * in Table were regressed including demographics, lifestyle and physical disease. Cognitive scores, blood indexes, and brain anatomy indexes were analyzed using general linear models and compared across groups after adjusting for distinguishing factors. Stepwise linear regression analysis was employed using follow-up cognitive function as dependent variable, with self-selected exercise (exercise = 1; non-exercise = 2) as independent variable. Logistic regression analysis was employed using dementia rate of 1 year follow-up as dependent variable, with self-selected exercise (exercise = 1; non-exercise = 2) as the independent variable. Covariates in these models included demographics (age, education, and sex), lifestyle (drinking, smoking, tea) and physical diseases (hypertension and diabetes). SPSS Version 17.0 software with a two-tailed *p*-values of 0.05 was used for all of the statistical analysis.

RESULTS

Cognitive function between self-selected exercise and self-selected non-exercise groups was compared. Demographics, physical disease, lifestyle, and cognitive scores for self-selected exercisers (n = 1372) and self-selected non-exercise (n = 702) groups are listed in **Table 1**. Differences were observed for demographics, lifestyle and physical disease, and the effects of confounding factors (signed by * in **Table 1**) were regressed. Through statistical analysis, higher baseline MMSE and MoCA scores were evident in self-selected exercise group compared to self-selected non-exercise group ($p < 0.05$), which was repeated at 1 year follow-up ($p < 0.05$) (**Figure 1**). Furthermore, we observed significant differences in visuospatial ability through baseline MoCA, baseline MMSE and 1 year follow-up MMSE tests ($p < 0.05$), whilst no significant difference in memory ability through baseline MoCA, baseline MMSE, 1 year follow-up MoCA tests ($p > 0.05$) except 1 year follow-up MMSE test ($p < 0.05$) between self-selected exercise and self-selected non-exercise groups ($p > 0.05$) was observed. Between ≥10 exercise years and 1–9 exercise years, we observed significant differences in baseline and 1 year follow-up MMSE and MoCA ($p < 0.05$). Furthermore, across all three groups (including ≥10 exercise years, 1–9 exercise years, and non-exercise groups), we observed significant differences in the baseline and 1 year follow-up of MMSE and MoCA ($p < 0.05$).

The incidence of dementia was 40 (4.4%) of the 915 elderly subjects in self-selected exercise group and 51 (10.5%) of 484 elderly subjects in self-selected non-exercise elderly group after 1 year follow-up ($p < 0.05$). However, no significant differences between groups that exercised at least 10 years or more and the 1–9 exercise years was observed ($p > 0.05$).

Stepwise linear regression was used to identify the risk factors for cognitive functions, showing that age ($B = -0.082$, $p = 0.000$), education ($B = 0.192$, $p = 0.000$), tea (tea = 1, non-tea = 2; $B = -0.316$, $p = 0.024$), baseline MMSE scores ($B = 0.654$, $p = 0.000$) and self-selected exercise (exercise = 1, non-exercise = 2; $B = -0.582$, $p = 0.000$) were associated with 1 year follow-up MMSE scores. Furthermore, age ($B = -0.112$, $p = 0.000$), education ($B = 0.278$, $p = 0.000$), (tea = 1, non-tea = 2; $B = -0.357$, $p = 0.047$), baseline MoCA score ($B = 0.626$, $p = 0.000$) and self-selected exercise (exercise = 1, non-exercise = 2; $B = -0.530$, $p = 0.005$) were associated with 1 year follow-up MoCA scores. Finally, logistic regression analysis revealed that education ($B = 0.089$, Wald = 11.632, $p = 0.000$) was positively associated with dementia occurrence after 1 year, however, age ($B = -0.079$, Wald = 25.964, $p = 0.000$), and self-selected non-exercise ($B = -0.829$, Wald = 13.386, $p = 0.001$) negatively affected the dementia rate at 1 year follow-up.

Blood Lipid Profiles Between Self-Selected Exercisers and Self-Selected Non-exercise Groups

Blood lipid tests were obtained from a total of 689 individuals in the self-selected exercise (n = 411) and self-selected non-exercise (n = 278) groups extracted from the whole database (**Table 2**). The effects of distinguishing factors between groups were regressed (signed by * in **Table 2**). Through regular statistical analysis, no changes in TG, CHOL, HDL and LDL levels was observed between self-selected exercise and self-selected non-exercise groups ($p > 0.05$). No significant differences in blood lipid levels was found in the group that exercised at least 10 years or more and the group that exercised from 1 to 9 year, or across the three groups ($p > 0.05$).

Brain Anatomy Between Self-Selected Exercise and Self-Selected Non-exercise Groups

Brain MR images were obtained from 141 individuals from the whole database. The demographics, physical disease, and lifestyle for the self-selected exercise (n = 82) and self-selected non-exercise (n = 59) groups were listed in **Table 3**. The effects of distinguishing factors were regressed (signed by * in **Table 3**). No significant differences in regional cortical thickness (a total of 68 brain region, not listed in table), cortical volume, and hippocampus volume, occurred between self-selected exercise and self-selected non-exercise, the group that exercised at least 10 or more years and the group that exercised 1–9 years, or across all three sub-groups ($p > 0.05$).

[1] http://surfer.nmr.mgh.harvard.edu/

TABLE 1 | Demography, life style, physical diseases, and cognitive function in the overall database of study participants in non-dementia elderly Chinese population.

Characteristic (Baseline)	**Self-selected exercise①** (*n* = 1372)		**Self-selected ②** non-exercise (*n* = 702)	**① vs.②** F/χ2 (*P*-value) (η_p^2)	**③ vs. ④** F/χ2 (*P*-value) (η_p^2)	**② vs. ③ vs. ④** F/χ2 (*P*-value) (η_p^2)
	≥10 years③ (*n* = 988)	**1–9 years④** (*n* = 384)				
Age(year)	70.86 ± 7.251	67.77 ± 6.560	70.96 ± 8.163	0.083(0.774)	101.876(0.000*)	45.792(0.000*)
Male/Female	478/510	163/221	304/398	2.184(0.139)	3.910(0.048*)	6.108(0.047*)
Education (year)	7.95 ± 4.648	7.4 ± 4.188	7.40 ± 4.873	3.383(0.066)	4.031(0.045*)	3.606(0.027*)
Smoking (Y/N)	283/705	126/258	212/490	0.033(0.855)	2.297(0.130)	2.324(0.313)
Drinking (Y/N)	221/767	99/285	130/572	6.311(0.012*)	1.801(0.180)	8.207(0.017*)
Tea (Y/N)	493/495	168/216	309/393	3.229(0.072)	4.187(0.041*)	7.429(0.024*)
Sleep disorder (Y/N)	136/852	66/318	114/588	0.827(0.363)	2.580(0.108)	3.334(0.189)
Hypertension (Y/N)	463/525	193/191	340/362	0.071(0.789)	1.280(0.258)	1.351(0.509)
Diabetes (Y/N)	151/837	70/314	108/594	0.182(0.670)	1.776(0.183)	1.980(0.372)
Baseline MoCA	22.07 ± 5.161	21.81 ± 5.128	21.44 ± 5.485	5.272(0.022*)(0.003)	11.028(0.001*)(0.008)	5.822(0.003*)(0.006)
Visual space	3.17 ± 1.512	3.21 ± 1.459	2.992 ± 1.671	6.113(0.013*)(0.003)	1.680(0.195)(0.001)	2.250(0.106)(0.002)
Memory	2.26 ± 1.723	2.14 ± 1.738	2.17 ± 1.680	0.648(0.421)(0.000)	9.669(0.002*)(0.007)	4.073(0.017*)(0.004)
Baseline MMSE	26.64 ± 3.058	26.51 ± 3.141	26.21 ± 3.343	7.335(0.007*)(0.004)	4.407(0.036*)(0.003)	3.993(0.019*)(0.004)
Visual space (0/1)	331/657	105/279	266/436	7.751(0.005*)	4.837(0.028*)	12.435(0.002*)
Memory	5.11 ± 1.010	5.18 ± 0.964	5.06 ± 1.051	2.537(0.111)(0.001)	0.005(0.945)(0.000)	0.662(0.516)(0.001)
Characteristic (1 year follow-up)	**Self-selected exercise①** (*n* = 915)		**Self-selected ②** non-exercise (*n* = 484)	**① vs. ②** F/χ2 (*P*-value) (η_p^2)	**③ vs. ④** F/χ2 (*P*-value) (η_p^2)	**② vs. ③ vs. ④** F/χ2 (*P*-value) (η_p^2)
	≥10 years③ (*n* = 664)	**1–9 years④** (*n* = 251)				
Age(year)	72.3 ± 7.322	67.71 ± 6.514	71.33 ± 8.198	0.429(0.513)	76.078(0.000*)	34.373(0.000*)
Male/Female	322/342	114/137	212/272	1.886(0.170)	0.691(0.406)	2.579(0.275)
Education (year)	8.04 ± 4.607	7.42 ± 4.103	7.29 ± 4.755	5.121(0.024*)	3.563(0.059)	4.270(0.014*)
Smoking (Y/N)	183/481	82/169	150/334	0.625(0.429)	2.311(0.128)	2.904(0.234)
Drinking (Y/N)	142/522	63/188	84/400	4.924(0.026*)	1.445(0.229)	6.456(0.040*)
Tea (Y/N)	336/328	114/137	208/276	4.893(0.027*)	1.959(0.162)	6.858(0.032*)
Sleep disorder (Y/N)	85/579	41/210	79/405	1.648(0.199)	1.915(0.166)	3.467(0.177)
Hypertension (Y/N)	311/353	141/110	238/246	0.006(0.936)	6.354(0.012*)	6.361(0.042*)
Diabetes (Y/N)	97/567	46/205	70/414	0.333(0.564)	1.910(0.167)	2.284(0.319)
1 Year MoCA	22.33 ± 5.442	22.30 ± 5.536	21.07 ± 6.172	8.742(0.003*)(0.006)	14.881(0.000*)(0.016)	7.120(0.001*)(0.010)
Visual space	3.17 ± 1.585	3.23 ± 1.529	2.94 ± 1.724	2.037(0.154)(0.001)	5.602(0.018*)(0.006)	1.212(0.298)(0.002)
Memory	2.40 ± 1.720	2.46 ± 1.739	2.26 ± 1.736	0.953(0.329)(0.001)	5.431(0.020*)(0.006)	1.112(0.329)(0.002)
1 Year MMSE	26.46 ± 3.624	26.5 ± 3.510	25.40 ± 4.567	16.039(0.000*)(0.011)	9.404(0.002*)(0.010)	9.640(0.000*)(0.014)
Visual space (0/1)	223/441	79/172	209/275	14.140(0.000*)	0.367(0.545)	14.489(0.001*)
Memory	5.15 ± 0.997	5.20 ± 0.924	4.95 ± 1.172	8.037(0.005*)(0.006)	2.600(0.107)(0.003)	4.675(0.009*)(0.007)
1 Year Dementia (Y/N)	32/632	8/243	51/433	19.787(0.000*)	1.161(0.281)	20.585(0.000*)

DISCUSSION

In this study, three major findings were presented. First, non-dementia elderly individuals with self-selected exercise showed significantly higher cognitive performance and a lower dementia rate of 1 year follow-up compared to those with self-selected non-exercise. Next, no significant difference in blood lipids occurred in response to self-selected exercise in elderly individuals. Finally, no significant differences in brain anatomy occurred between self-selected exercise and self-selected non-exercise in elderly individuals.

This study highlighted higher cognitive performance in self-selected exercisers without dementia, supporting previous observational studies (Hamer and Chida, 2009; Prince et al., 2013; Livingston et al., 2017). Stepwise linear regression analysis also suggested that, besides age and education, exercise was an important risk factor for the occurrence of dementia in the elderly. A meta-analysis of prospective studies supported this

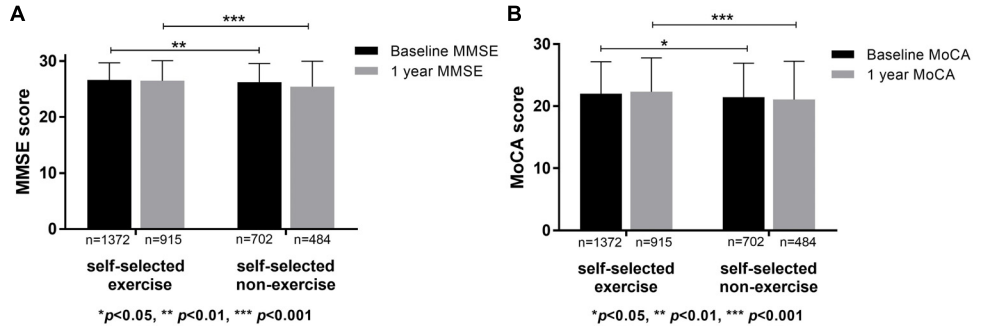

FIGURE 1 | Cognitive functions in self-selected exercise and self-selected non-exercise groups. **(A)** Significant differences were apparent of baseline and 1 year follow-up MMSE scores between self-selected exercise and self-selected non-exercise groups. **(B)** Significant differences of baseline and 1 year follow-up MoCA scores were apparent between self-selected exercise and self-selected non-exercise groups.

TABLE 2 | Demography, life style, physical diseases, and lipid profile of study participants in the database subgroup on a non-dementia elderly Chinese population.

Characteristic	Self-selected exercise① (*n* = 411)		Self-selected non-exercise② (*n* = 278)	① vs. ② F/χ^2(*P*-value) (η_p^2)	③ vs. ④ F/χ^2(*P*-value) (η_p^2)	② vs.③ vs. ④ F/χ^2(*P*-value) (η_p^2)
	≥10 years③ (*n* = 306)	1–9 years④ (*n* = 105)				
Age(year)	72.42 ± 7.518	68.04 ± 7.071	71.27 ± 8.147	0.002(0.961)	27.337(0.000*)	12.603(0.000*)
Male/Female	137/169	45/60	119/159	0.147(0.701)	0.116(0.733)	0.263(0.877)
Education (year)	8.01 ± 4.440	8.90 ± 3.799	7.54 ± 4.527	4.168(0.042*)	3.433(0.065)	3.731(0.024*)
Smoking (Y/N)	77/229	28/77	76/202	0.275(0.600)	0.093(0.761)	0.366(0.833)
Drinking (Y/N)	60/246	18/87	46/232	0.664(0.415)	0.309(0.578)	0.986(0.611)
Tea (Y/N)	142/164	36/69	101/177	3.351(0.067)	4.677(0.031*)	8.116(0.017*)
Sleep disorder (Y/N)	49/257	20/85	59/219	2.156(0.142)	0.515(0.473)	2.632(0.268)
Hypertension (Y/N)	144/162	55/50	135/143	0.001(0.971)	0.887(0.346)	0.888(0.641)
Diabetes (Y/N)	48/258	26/79	36/242	3.159(0.076)	4.362(0.037*)	7.958(0.019*)
Blood Lipid Profile						
TG (mmol/L)	1.83 ± 1.347	1.92 ± 1.566	1.73 ± 1.237	0.974(0.324)(0.001)	0.039(0.844)(0.000)	0.527(0.591)(0.002)
CHOL (mmol/L)	4.84 ± 1.083	4.80 ± 1.035	4.88 ± 1.088	0.196(0.658)(0.000)	0.160(0.690)(0.000)	0.102(0.903)(0.000)
HDL (mmol/L)	1.21 ± 0.313	1.21 ± 0.361	1.23 ± 0.340	0.023(0.879)(0.000)	0.196(0.659)(0.000)	0.266(0.766)(0.001)
LDL (mmol/L)	2.89 ± 0.848	2.85 ± 0.911	2.97 ± 0.8883	1.356(0.245)(0.002)	0.556(0.456)(0.001)	0.668(0.513)(0.002)

TG, triglyceride; CHOL, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein.

concept and suggested that physical exercise lowered the risk of cognitive deficits and dementia by up to 38% (Sofi et al., 2011), which supported the findings of this research study. After 1 year follow-up, the rate of dementia occurrence in the self-selected exercise group (4.4%) was significantly lower than the self-selected non-exercise group (10.5%) in the present study. Our cohort was non-dementia aging Chinese population including normal cognitive and mild cognitive impairment elderly. Most subjects converting into dementia with 1 year were mild cognitive impairments elderly. Furthermore, we found better visuospatial ability with self-selected exercisers, which was similar with some previous publications. Aerobic exercise may have a positive effect on improving a potential benefit on visuospatial domain of cognition and et al., in stroke survivors (Zheng et al., 2016). Improvements in visual ability and et al., in the stabilization exercise training suggest exercise for the treatment of idiopathic scoliosis to improve internal body orientation (Yagci et al., 2018). A serious of RCTs reported that physical exercise did not improve cognitive function or lower the risk of dementia (Legault et al., 2011; Barnes et al., 2013; Sink et al., 2015). This finding might be related to the short study period. All of the subjects involved in the present study maintained exercise for at least 1 year and 97% of the subjects maintained exercise for two or more years. The longest intervention duration was less than 2 years in the previous RCT (Lam et al., 2011), which suggested that exercise duration of two or more years was an important influencing factor on cognition. Significant differences in cognition function were observed including a baseline and 1 year follow-up MMSE and MoCA of the group that exercised 10 or more years and the group that exercised 1–9 years, demonstrating that the duration of physical exercise affected cognition (Varma et al., 2015). However, no differences in dementia occurrence between the 10 or more years and 1—9 exercise years groups, suggesting that the cut-off value of 10 years did not influence the incidence of dementia.

TABLE 3 | Demography, life style, physical diseases, and brain anatomy of study participants in the database subgroup on a non-dementia elderly Chinese population.

Characteristic	Self-selected exercise① (*n* = 82)		Self-selected non-exercise② (*n* = 59)	① vs. ② F/χ^2(*P*-value) (η_p^2)	③ vs. ④ F/χ^2(*P*-value) (η_p^2)	② vs. ③ vs. ④ F/χ^2(*P* value) (η_p^2)
	≥10 years③ (*n* = 55)	1–9 years④ (*n* = 27)				
Age (year)	69.11 ± 6.839	65.41 ± 5.719	68.25 ± 7.473	0.092(0.762)	5.881(0.018*)	2.636(0.075)
Male/Female	30/25	11/16	29/30	0.010(0.921)	1.380(0.240)	1.390(0.499)
Education (year)	9.84 ± 3.207	10.19 ± 3.352	9.93 ± 3.3398	0.001(0.973)	0.208(0.650)	0.101(0.904)
Smoking (Y/N)	13/42	5/22	21/38	3.191(0.074)	0.277(0.599)	0.454(0.797)
Drinking (Y/N)	11/44	5/22	9/50	0.426(0.514)	0.025(0.874)	0.454(0.797)
Tea (Y/N)	24/31	8/19	23/36	0.000(0.996)	1.493(0.222)	1.493(0.474)
Sleep disorder (Y/N)	2/53	5/22	13/46	5.135(0.023*)	5.137(0.023*)	8.430(0.015*)
Hypertension (Y/N)	27/28	14/13	28/31	0.089(0.766)	0.055(0.814)	0.144(0.931)
Diabetes (Y/N)	7/48	5/22	9/50	0.010(0.919)	0.486(0.486)	0.490(0.783)
Volume (cm^3)						
Total volume	1459.20 ± 141.52	1422.33 ± 158.53	1475.69 ± 143.472	2.072(0.152)(0.015)	0.556(0.458)(0.007)	1.390(0.253)(0.020)
Cortex volume	417.28 ± 37.389	408.99 ± 38.037	420.35 ± 38.783	0.057(0.811)(0.000)	0.825(0.367)(0.011)	0.034(0.967)(0.000)
Left hippocampus	3.64 ± 0.338	3.66 ± 0.375	3.65 ± 0.472	0.281(0.597)(0.002)	0.001(0.975)(0.000)	0.600(0.550)(0.009)
Right hippocampus	3.88 ± 0.404	3.92 ± 0.398	3.84 ± 0.426	2.175(0.143)(0.016)	0.000(0.984)(0.000)	1.713(0.184)(0.025)

Physical exercise had been shown to have a beneficial impact on dyslipidemia and numerous studies had reported that physical exercise combined with weight loss significantly reduced blood CHOL, LDL, and TG, while improving HDL (O'Donovan et al., 2005; Pattyn et al., 2013; Gordon et al., 2014). However, we observed no significant difference in blood lipid profiles between self-selected exercise and self-selected non-exercise groups. Interventional research to directly assess the impact of training intensity on lipid profiles by controlling training volume showed that significant improvements occurring only in high-intensity compared to moderate intensity groups (Mann et al., 2014). The subjects included in self-selected exercise groups maintained moderate and/or vigorous exercise intensity, not high intensity exercise.

No difference in brain anatomy including regional cortical thickness, total cortical volume, hippocampus volume was found between self-selected exercise and self-selected non-exercise groups. Other studies found that exercise training increased gray matter volume in the prefrontal lobe (Colcombe et al., 2006), temporal lobe, and hippocampus (Erickson et al., 2011). In this study, no region was found to be significantly thicker or larger in the self-selected exercise group. This might be due to the variety of exercise types that the test subjects adopted in the different cohorts. The previous studies demonstrated that significant increases in brain volume were found as a function of aerobic fitness training but were not found in stretching and toning (non-aerobic) (Colcombe et al., 2006; Erickson et al., 2010). In the current research, the exercise aerobic and anaerobic exercise types were not distinguished, which could cause the negative brain anatomy results.

There were several study limitations. Follow-up tests using MR imaging and blood indexes were not performed, and therefore, could not directly show causality of exercise on brain anatomy and lipid profiles, either beneficial or harmful. A lack of detailed information regarding oxygen consumption, heart rate and exercise types also limited the description of exercise status. Self-selected exercise based on self-reported by subjects, and several factors including education, lifestyle and comorbid diseases might be association with self-selected exercise, which is a possible bias of the study. The sample size of MR images was significantly smaller than the overall database since only elderly subjects in Shanghai were able to receive MR imaging scans. The follow-up duration was only 1 year and inadequate to reflect dementia occurrence, and we would go on research the cohort in the future.

CONCLUSION

The study results demonstrate that physical exercise has beneficial effects on cognition, particularly visuospatial function, and lowers the risk of dementia. No differences in the blood lipids and brain anatomy were observed between self-selected exercise and self-selected non-exercise groups in the Chinese aging cohort.

CONSENT TO PUBLICATION

All subjects also gave written informed consent for the publication of this case report.

DATA AVAILABILITY

The data supporting our findings can be requested by email to the correspondence author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the "Shanghai Mental Health Center ethical standards committee on human experimentation" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "Shanghai Mental Health Center ethical standards committee."

AUTHOR CONTRIBUTIONS

SL analyzed the data and wrote the manuscript. YY, QQ, LW, NJ, and ZJ evaluated the subjects and collected the data. XS and LX designed the experiment and monitored the quality of the experiment.

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Sex-Dependent Differences in Physical Exercise-Mediated Cognitive Recovery Following Middle Cerebral Artery Occlusion in Aged Rats

*Charles H. Cohan†, Mehdi Youbi†, Isabel Saul, Alex A. Ruiz, Concepcion C. Furones, Pujan Patel, Edwin Perez, Ami P. Raval, Kunjan R. Dave, Weizhao Zhao, Chuanhui Dong, Tatjana Rundek, Sebastian Koch, Ralph L. Sacco and Miguel A. Perez-Pinzon**

Peritz Scheinberg Cerebral Vascular Disease Research Laboratory, Department of Neurology, University of Miami Miller School of Medicine, Miami, FL, United States

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Aderbal S. Aguiar Jr., Federal University of Santa Catarina, Brazil

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***Correspondence:**
Miguel A. Perez-Pinzon
perezpinzon@miami.edu; perezpinzon@med.miami.edu

†These authors have contributed equally to this work as co-first authors

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Stroke remains a leading cause of death and disability in the United States. No current treatments exist to promote cognitive recovery in survivors of stroke. A previous study from our laboratory determined that an acute bout of forced treadmill exercise was able to promote cognitive recovery in 3 month old male rats after middle cerebral artery occlusion (MCAo). In this study, we tested the hypothesis that 6 days of intense acute bout of forced treadmill exercise (physical exercise – PE) promotes cognitive recovery in 11–14 month old male rats. We determined that PE was able to ameliorate cognitive deficits as determined by contextual fear conditioning. Additionally, we also tested the hypothesis that PE promotes cognitive recovery in 11–13 month old reproductive senescent female rats. In contrast to males, the same intensity of exercise that decrease cognitive deficits in males was not able to promote cognitive recovery in female rats. Additionally, we determined that exercise did not lessen infarct volume in both male and female rats. There are many factors that contribute to higher stroke mortality and morbidities in women and thus, future studies will investigate the effects of PE in aged female rats to identify sex differences.

Keywords: stroke, brain focal ischemia, reproductive senescent female, cognition, treadmill exercise, contextual fear conditioning

INTRODUCTION

Stroke is the 5th leading cause of death in the United States and a leading cause of disability. Those that survive strokes are often left with both motor and cognitive impairments. Currently, there is no accepted neurological treatments to promote cognitive recovery in clinical practice. One potential strategy to promote cognitive recovery after a stroke is the use of physical exercise.

Physical exercise has beneficial effects in improving cognition in children (Davis et al., 2011) and the elderly (Blanchet et al., 2018; Kennedy et al., 2018) after traumatic brain injury (Griesbach et al., 2004) and post stroke (Marzolini et al., 2013). The optimal amount of exercise or exercise type that promotes recovery is not well defined. A previous study from our laboratory

indicated that an acute bout of forced treadmill exercise (30 min a day for 6 days at a speed of 10–12 m/min) promoted cognitive recovery following both cardiac arrest and stroke in 3 month old male rats (Stradecki-Cohan et al., 2017). However, stroke is an injury that commonly occurs in the elderly. This is why the STAIR criteria emphasize the importance of testing pre-clinical therapies in aged animals and also to determine sex differences (Albers et al., 2011). In this study, we aimed to investigate the ability of forced treadmill exercise to promote cognitive recovery in 11–14 month old male and 11–13 months old female rats. Furthermore, we investigated the modifying effects of exercise on post stroke infarct volume and on sex dependent differences in survival.

MATERIALS AND METHODS

Animal Care

All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals and approved by the University of Miami Institutional Animal Care and Use Committee. Retired breeder Sprague – Dawley rats of both sexes were purchased from Charles River and were housed within the division of veterinary resources facility for the entire length of the experiment. Animals were allowed access to food and water *ad libitum*. Prior to exposure to any experimental paradigm, animals were handled by researchers for a 3 day period in order to acclimate them to human touch to reduce stress and discomfort throughout the experimental procedures. All animals were fasted overnight prior to surgery. The age of retired breeder male rats was 11–14 months at the time of MCAO.

Female Reproductive Senescence Determination

The estrous cycles of retired breeder rats were monitored for ∼20 days prior to experimentation by daily examination of vaginal smears (de Rivero Vaccari et al., 2016). Retired breeder rats that remained in constant diestrous were considered reproductively senescent (RS) (Selvamani and Sohrabji, 2010; de Rivero Vaccari et al., 2016). The age of RS rats was 11–13 months at the time of MCAO.

Transient Middle Cerebral Artery Occlusion (MCAO)

Age matched male and female rats were exposed to MCAO. MCAO was achieved with intraluminal suture blockage of the middle cerebral artery for 90 min as described in previous publications (Belayev et al., 1996; Lin et al., 2014). Physiological parameters were maintained within normal limits through the surgery.

Forced Treadmill Exercise

Three days prior to undergoing MCAO surgery, rats were exposed to the treadmill for a 3 day period, in order to acclimate themselves and reduce stress in later behavioral experiments. Three days following MCAO animals began daily exercise training for 6 days (Stradecki-Cohan et al., 2017). All animals were placed on the treadmill for a 2 min warm up at a speed of 5 m/min. After the warmup period rats ran on the treadmill for a 30 min period. The exercise speeds for each group were as follows; no exercise (0 m/min), mild exercise (6 m/min), moderate exercise (9 m/min), and intense exercise (12 m/min). In our previous study, 3 month old male rats were able to run at a speed of 18 m/min for 30 min (Stradecki-Cohan et al., 2017). However, 12 month old rats were unable to run at this speed after MCAO. Therefore, we reduced the intense exercise group to 12 m/min. For female rats, only intense exercise (12 m/min) paradigm and a no exercise control group were tested. An electric shock grid (0.2 mA) was active at the back of the treadmill so that if the animal stopped running during the experiment a light electric shock was delivered to the animal as motivation to continue running. No animals were excluded for their inability to run on the treadmill in this experiment. We excluded animals that suffered respiratory dysfunction, surgery complications or died during the course of the study.

Contextual Fear Conditioning

Twenty days following MCAO injury, rats were subjected to contextual fear condition test (Zelikowsky et al., 2012; Cohan et al., 2015; Stradecki-Cohan et al., 2017). Briefly, rats were brought into the fear conditioning room 20 min prior to testing. Animals were then placed into the operant conditioning chamber (Colbourne Instruments, United States). Animals were allowed to explore the chamber for 340 s and then animals received a 2 s 1.5 mA shock. Animals were then removed from the chamber after 30 s. The following day, animals were again returned to the same room 20 min prior to testing. Rats were then placed in the fear conditioning chamber for an 8 min period. The amount of time spent freezing during an 8 min period was measured using FreezeFrame software.

Infarct Volume Measurement

Three weeks following MCAO, animals were perfused with saline and then with a formaldehyde, acetic acid, and methanol solution (FAM; 10:10:80). Brains were then embedded with paraffin and 10 μm thick sections were obtained. Infarct volume measurements were adapted from previous publications (Belayev et al., 2005). Briefly, the same nine coronal sections were selected for every animal (Bregma levels 5.2, 2.7, 1.2, −0.3, −1.3, −1.8, −3.8, −5, and −7.3). Infarcted area was then measured on each section using MCID software. Infarct volume was then determined similar to previously used methods (Belayev et al., 2005).

Statistical Analysis

All figures are displayed as means with standard error of the mean (SEM) error bars. We used the Student's *t*-test for comparisons of two groups, as well as Chi square test, or One-way ANOVA measurements with a Bonferroni *post hoc* test as indicated within the text. We used a Fischer exact test to determine the differences in infarct volumes between the specific brain regions.

RESULTS

Physiological Parameters Consistent for Aged Male and Female Rats Except Body Weight

In order to determine that the severity of the injury given is uniform between animals, a number of physiological parameters were measured for both aged male (**Table 1**) and aged female rats (**Table 2**). Body weight, cranial temperature, rectal temperature, blood pH, pCO_2 pO_2, MABP, and blood glucose were measured before and after MCAO. We attempted to maintain physiological parameters in normal range throughout the experiment (**Tables 1**, **2**).

Both males and females underwent the same experimental paradigm. Survival rates throughout the paradigm were observed for aged males and female animals prior to exercise (**Figure 1A**). Male and female animals demonstrated a three week survival rate of 62% (54/89) and 79% (31/39), respectively. All mortality except for two animals (one male and one female) occurred within 4 days of MCAO surgery (prior to exercise treatment). The survival rate was higher in aged females than in aged males ($P < 0.05$, Chi Square, $n = 134$, 89 male, 39 female). No animal in the sham MCAO surgery group died (male $n = 10$ and female = 10).

TABLE 1 | MCAO physiological parameters for male rats belonging to specific exercise and no exercise groups.

Group	Variable	Before MCAO	After MCAO
Sham ($n = 10$)	Body weight (g)	630 ± 35	
	Cranial temp (°C)	36.4 ± 0.17	36.8 ± 0.12
	Rectal temp (°C)	36.5 ± 0.28	37.0 ± 0.03
	pH	7.37 ± 0.07	7.40 ± 0.06
	pCO_2 (mmHg)	38.9 ± 6.56	36.4 ± 4.69
	pO_2 (mmHg)	119 ± 18	144 ± 36
	MABP (mmHg)	122 ± 9	111 ± 11
	Blood glucose (mg/dl)	142 ± 26	155 ± 50
MCAO + No exercise ($n = 11$)	Body weight (g)	634 ± 51	
	Cranial temp (°C)	36.4 ± 0.29	36.6 ± 0.35
	Rectal temp (°C)	36.4 ± 0.25	36.8 ± 0.29
	pH	7.40 ± 0.04	7.38 ± 0.05
	pCO_2 (mmHg)	38.8 ± 3.13	39.0 ± 5.83
	pO_2 (mmHg)	127 ± 31	131 ± 17
	MABP (mmHg)	117 ± 7	105 ± 10
	Blood glucose (mg/dl)	167 ± 36	164 ± 34
MCAO + Mild exercise ($n = 10$)	Body weight (g)	617 ± 68	
	Cranial temp (°C)	36.3 ± 0.16	36.8 ± 0.60
	Rectal temp (°C)	36.5 ± 0.25	37.0 ± 0.43
	pH	7.40 ± 0.07	7.43 ± 0.05
	pCO_2 (mmHg)	37.5 ± 3.65	35.6 ± 5.53
	pO_2 (mmHg)	120 ± 35	132 ± 23
	MABP (mmHg)	117 ± 11	116 ± 15
	Blood glucose (mg/dl)	138 ± 25*	144 ± 40
MCAO + Moderate exercise ($n = 11$)	Body weight (g)	632 ± 65	
	Cranial temp (°C)	36.2 ± 0.19	36.7 ± 0.55
	Rectal temp (°C)	36.5 ± 0.17	37.0 ± 0.50
	pH	7.41 ± 0.04	7.39 ± 0.04
	pCO_2 (mmHg)	40.2 ± 3.69	39.4 ± 4.04
	pO_2 (mmHg)	121 ± 35	132 ± 31
	MABP (mmHg)	120 ± 7	112 ± 18
	Blood glucose (mg/dl)	145 ± 41	170 ± 48
MCAO + Intense exercise ($n = 11$)	Body weight (g)	627 ± 66	
	Cranial temp (°C)	36.3 ± 0.25	36.6 ± 0.24#
	Rectal temp (°C)	36.3 ± 0.19	36.7 ± 0.16#,#
	pH	7.40 ± 0.04	7.39 ± 0.04
	pCO_2 (mmHg)	40.9 ± 3.93	37.4 ± 3.07
	pO_2 (mmHg)	136 ± 26	129 ± 24
	MABP (mmHg)	115 ± 15	110 ± 14
	Blood glucose (mg/dl)	141 ± 37	141 ± 41

*$p < 0.05$ vs. no exercise group. #$p < 0.05$ vs. sham group. #,#$p < 0.001$ vs. sham group.

Forced Treadmill Exercise Improves Cognitive Recovery in Aged Male but Not in Aged Female Rats

To determine the importance of an acute bout of physical exercise on long term cognitive outcomes in aged male and female animals, we employed a previously used paradigm developed in our laboratory (Stradecki-Cohan et al., 2017). This paradigm uses an acute bout of forced treadmill training, followed by assessing cognitive recovery and infarct volume at around 3 weeks post-MCAO (**Figure 1A**). In order to determine cognitive recovery a contextual fear conditioning task measuring freezing response was determined at the end of the 3 week survival period (**Figure 1B**). Previous protocols found that contextual fear conditioning was a sensitive measure of determining cognitive recovery in rats that had undergone cerebral ischemia (Cohan et al., 2015; Stradecki-Cohan et al., 2017).

In aged rat males, day 1 prior to the shock, freezing response to the context was measured in order to determine whether there was any baseline mobility or anxiety toward the context for aged males (**Figure 2A**). Rats that underwent sham surgery ($n = 10$) froze 10.7 ± 4.9% of the time, whereas animals that underwent MCAO but had no exercise ($n = 7$) froze 6.5 ± 0.8% of the time. Mild exercise animals ($n = 10$), moderate exercised animals ($n = 9$), and animals that underwent intense exercise ($n = 10$) froze 7.6 ± 1.9%, 4.3 ± 0.8%, and 10.8 ± 3.4% of the time, respectively. There were no observed significant differences between any of the groups for male rats on day 1 (1 way ANOVA, Bonferroni *post hoc*). Following contextual fear conditioning paradigm, rats were then tested again 24 h later. Rats that underwent sham surgery froze 41.7 ± 10.2% of the time. Intensely exercised rats froze 57.8 ± 9.0% of the time, which was significantly higher than animals that received an MCAO injury but did not exercise, which froze 14.5 ± 2.2% of the time ($P < 0.05$, 1 way ANOVA, Bonferroni *post hoc*). There was no significant difference between the mild exercise group (froze 26.2 ± 6.3% of the time), moderate exercise group (34.4 ± 10.2% of the time), and the no exercise group (14.5 ± 2.2% of the time).

Based on these findings, we proceeded to test the hypothesis that the intense rate of exercise also improves contextual fear

TABLE 2 | MCAO physiological parameters for female rats belonging to exercise and no exercise groups.

Group	Variable	Before MCAO	After MCAO
Sham (n = 10)	Body weight (g)	353.4 ± 31	
	Cranial temp (°C)	36 ± 0.49	36.77 ± 0.46
	Rectal temp (°C)	36 ± 0.24	36 ± 0.39
	pH	7.4 ± 0.03	7.42 ± 0.04
	pCO_2 (mmHg)	41.2 ± 2.7	36.6 ± 1.35
	pO_2 (mmHg)	152.5 ± 30.7	136.25 ± 18.3
	MABP (mmHg)	142.7 ± 8.6	127 ± 17
	Blood glucose (mg/dl)	172.56 ± 38	164.12 ± 56
MCAO + No exercise (n = 10)	Body weight (g)	363.4 ± 43	
	Cranial temp (°C)	36 ± 0.46	36.8 ± 0.5
	Rectal temp (°C)	35.5 ± 1.25	36 ± 0.43
	pH	7.43 ± 0.07	7.37 ± 0.04
	pCO_2 (mmHg)	38.18 ± 2.4	42.52 ± 1.6
	pO_2 (mmHg)	125.41 ± 32.04	120.8 ± 16.76
	MABP (mmHg)	144 ± 18	134 ± 30
	Blood glucose (mg/dl)	172.8 ± 31	196.1 ± 23
MCAO + Exercise (n = 11)	Body weight (g)	365.1 ± 44	
	Cranial temp (°C)	36 ± 0.23	36.8 ± 0.24
	Rectal temp (°C)	36 ± 0.1	36 ± 0.55
	pH	7.42 ± 0.03	7.38 ± 0.02
	pCO_2 (mmHg)	37.8 ± 3.83	41.33 ± 2.68
	pO_2 (mmHg)	111.95 ± 37	104.8 ± 11.03
	MABP (mmHg)	139.47 ± 9.9	136.0 ± 12.7
	Blood glucose (mg/dl)	175 ± 20	198.75 ± 10.26

conditioning response in aged female rats. Rats on Day 1 of the context prior to shock exhibited minimal freezing. Female rats that underwent sham surgery froze 2.5 ± 0.57% of the time. Female rats that underwent MCAO surgery but did not exercise froze for 4.1 ± 2.3% of the time as compared with rats that received exercise post MCAO who froze 1.8 ± 0.4% of the time ($P > 0.05$, Students T-test). When animals were returned to the context on the second day to assess cognitive recovery, freezing results were different for female rats (**Figure 2D**), as compared to aged male rats. Female rats that underwent sham surgery froze 28.63 ± 5.7% of the time on second day. For female animals, the exercise intensity that promoted cognitive recovery in males showed no significant difference in freezing response (26.1 ± 6.4% of the time) compared to a no exercise MCAO control (17.7 ± 4.9% of the time). This difference indicates that forced exercise in aged males and females have different outcomes on promoting cognitive recovery following MCAO injury.

Forced Treadmill Exercise Does Not Reduce Infarct Volume in Aged Male or Female Rats

One potential explanation for the improved behavioral outcome is the role of exercise in promoting a reduction in the infarcted area (Dang et al., 2011). This hypothesis was based on the fact that exercise can promote increased levels of neurotrophic factors such as BDNF, which can help reduce infarct volume over time (Zhang and Pardridge, 2006). Thus, we measured the infarcted area in each group at the three-week time point after MCAO as a secondary outcome (**Figure 3**). Sham male and female animals showed no infarction (data not shown). For aged male rats, animals that underwent MCAO + no exercise had an infarct volume of 24.64 ± 10.31 mm^3. This volume was not significantly different in the mild exercise group (34.03 ± 9.81 mm^3), the moderate exercise group (18.93 ± 9.15 mm^3), or the intense exercise group (30.77 ± 12.69 mm^3) ($P > 0.05$, one way ANOVA, Bonferroni *post hoc*) (**Figure 3A**). For aged female rats, animals that did not exercise displayed an infarct volume of 39.99 ± 9.07 mm^3, which also was not significantly different from animals that underwent intense exercise 44.68 ± 12.05 mm^3 (**Figure 3B**). Exercise did not significantly increase total volume of infarct in either males or females.

Specific Cortical and Striatal Areas Show Increased Injury in Aged Male Rats

Although we determined that there was no significant difference in overall volume changes, we wanted to investigate region specific changes in infarcted region. Region specific changes may explain the observable cognitive recovery promoted by intense exercise in the aged male rat cohort (**Figure 4**). Interestingly, intense exercise was protective in a number of regions when compared to the no exercise group (**Figures 4B,C**). There were significant reductions in infarct volume found within the thalamus, and the striatum (**Figures 4B,C**), the globus pallidus, and within the basal nucleus of Meynert (**Figures 4B,C**) in intense exercise group compared to no exercise group. These changes highlight specific areas of focus that may explain some of observed cognitive performance on the contextual fear conditioning test 3 weeks after injury.

DISCUSSION

Stroke affects mostly aged individuals (Benjamin et al., 2018). Aging may worsen outcomes by increasing the severity of the deficits (Rosen et al., 2005). Preclinical work investigating the best outcomes after stroke in aged individuals have determined that many therapies that are effective in young animals are less effective in aged animals. Thus, the main focus of the paper is to determine whether forced treadmill exercise ameliorates cognitive impairment in aged animals of both the sexes after focal cerebral ischemia.

In our previous study, we showed that after MCAO, young male rats (3 month old) subjected to moderate exercise (10 m/min) for 6 days (as in the current study) had a significant enhancement in duration of contextual freezing compared to non-exercised rats (Stradecki-Cohan et al., 2017) indicating improved cognitive encoding/recall. In that study, we observed that moderate exercise (10 m/min) improved contextual fear conditioning response 21 days after MCAO. However, we also saw trends for improvement at the mild (6 m/min) and intense (18 m/min) exercise. As we began our studies with the aged male rats, we noticed that these rats were unable to run the treadmill at 18 m/min and that 12 m/min was the highest intensity we could use consistently in this age cohort. This level of exercise

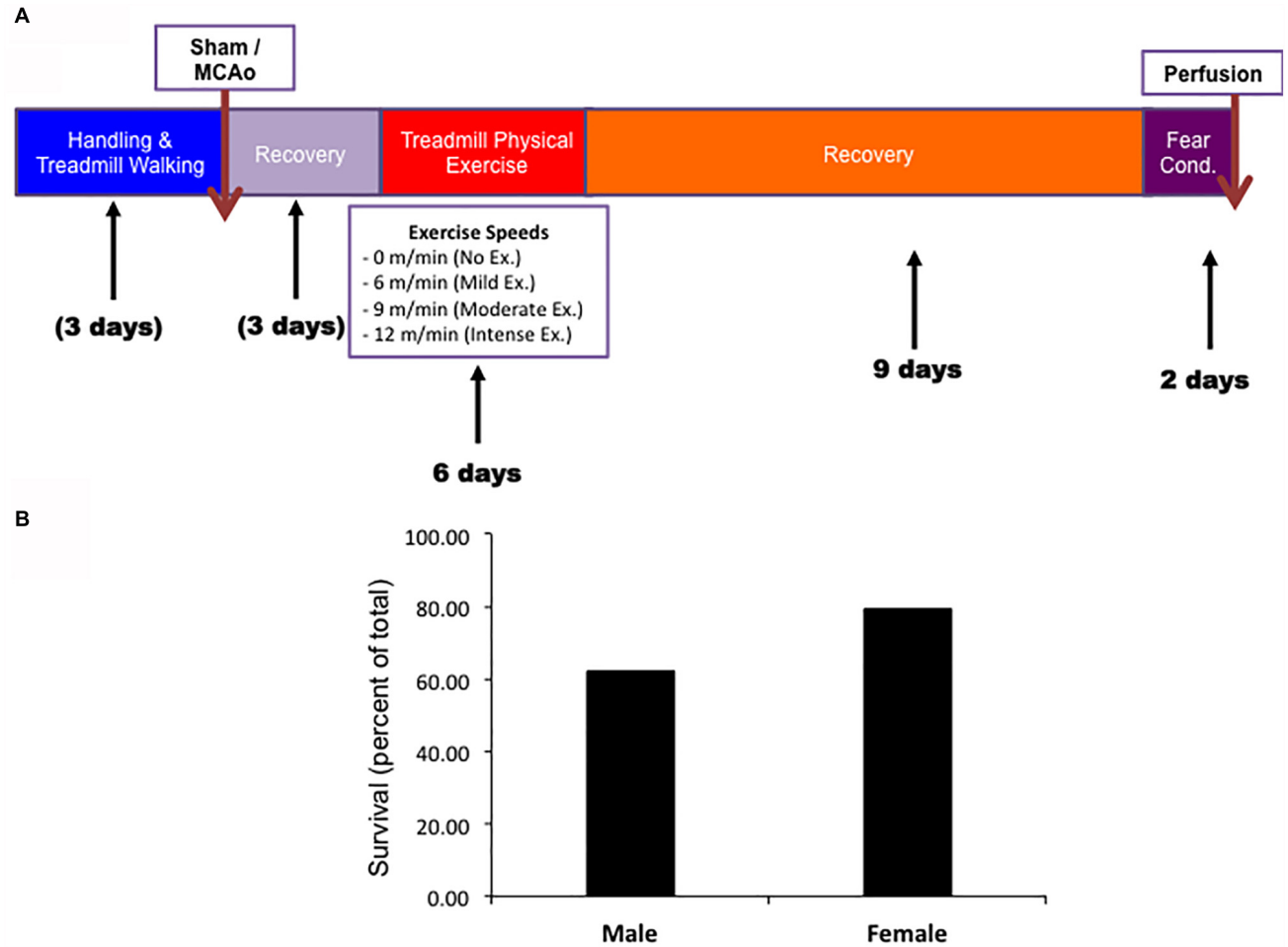

FIGURE 1 | (A) Timeline of experimental procedures for both male and female experimental groups. **(B)** Survival rates for both male and female animals.

intensity was sufficient to improve recall in the contextual fear conditioning paradigm. Lower exercise intensities however, were unable to ameliorate contextual recall deficits. For example, in our previous study, 10 m/min PE intensity in young rats was able to ameliorate contextual recall deficits, but in 12 month old rats, 9 m/min had no effect. These results further stress the importance of testing stroke treatments in aged animals that more closely resemble the aged human population that suffers strokes. As we continue our studies into even older cohorts of animals, we will probably encounter additional challenges.

A key finding from our study was that the intense exercise paradigm that improved post-stroke cognition in aged male rats was not effective in improving cognitive outcomes in aged matched RS female rats. The cause of reproductive senescence in female rodents is the depletion of ovarian oocytes reserve leading to the inability of ovaries to produce hormones, progesterone, and estrogen. Especially, estrogen is crucial for neurogenesis (Galea et al., 2006), spinal plasticity (Barha and Galea, 2010; Spencer-Segal et al., 2012), long-term potentiation (Liu et al., 2008), and cognition (Barha et al., 2010). In contrast to females, in male rats, reproductive aging is not well defined, and occurs much later in lifespan resulting in decreased fertility. Since the current study used RS female and age matched male rats, the effects of estrogen on cognition might be weaned off while those of testosterone (Bimonte-Nelson et al., 2003; Jian-xin et al., 2015) may persist and reflect on improved post-stroke cognition in exercised male rats. In middle-aged rats, similar to men, exercise training increases testosterone, sex-hormone binding globulin, and endothelial nitric oxide (e-NOS) (Zmuda et al., 1996; Seo et al., 2018). Therefore, the observed improvement in post-stroke cognition in exercised male rats could be due to enhanced cerebral blood flow and testosterone mediated effects. A study testing the effects of exercise on skeletal muscles, demonstrated that exercise training improves mitochondrial function oxidative capacities in both male and female rats, but it is more pronounced in males (Farhat et al., 2017). In the case of female rats, the effectiveness of regular and moderate intensity physical exercise is age-dependent (Marosi et al., 2012) and RS female may require extended period of physical activity before the benefits on memory become apparent (Sager et al., 2018), which indicates that it is important to consider titration of exercise for female animals.

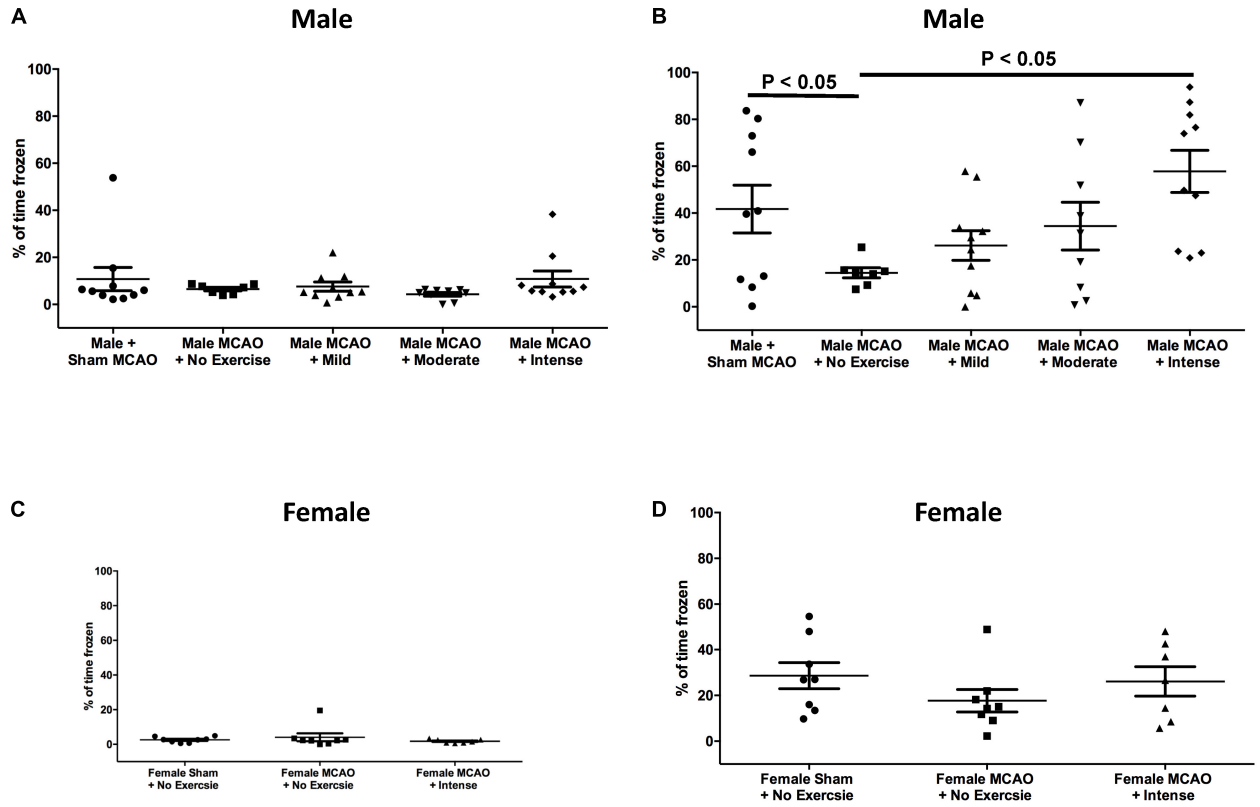

FIGURE 2 | Contextual fear conditioning as a measure of spatial memory performance 3 weeks following MCAO injury in aged male and female rats. **(A)** Day 1 as a percent of time spent frozen prior to exposure of shock stimulus for aged male rats. There were no significant differences between groups (One way ANOVA, Bonferroni *post hoc*). **(B)** Day 2, 24 h following exposure to shock in the same context for aged males. Animals that underwent intense exercise froze significantly more than animals that received no exercise ($P < 0.05$, One way ANOVA, Bonferroni *post hoc* test). **(C)** Day 1 as a percent of time spent frozen prior to exposure of shock stimulus for aged female rats (Students *t*-test). **(D)** Aged female rats at the same time point with or without intense exercise. There was no observed benefit for aged female animals.

Previous studies tested a number of different exercise paradigms to promote functional recovery. In a different paradigm than the one presented in our study, forced treadmill exercise paradigms administered prior to MCAO showed some improvement in the ability to protect against injury (Hayes et al., 2008). In another paradigm, voluntary wheel running experiments promoted some benefit post stroke, showing an improvement in both motor outcomes, and the molecular profile of peri-infarct tissue reduction (Mizutani et al., 2011). Forced physical exercise, as the paradigm we used in our study, has been previously demonstrated to promote beneficial behavioral and histological outcomes after stroke. An earlier study using young rats reported that forced treadmill exercise of 20 and 30 m/min day for 14 consecutive days post-MCAO was able to lower infarct volume as well as improve motor function when evaluated on day 14 post-reperfusion (Chang et al., 2011). This study did not test for cognitive improvement. Another study in young male rats, evaluated the effect of forced physical training (rota-rod at a speed of 12 m/min for 40 min daily for 14 consecutive days) post-MCAO on functional recovery. Significant improvement on modified neurological severity scores (mNSS) was observed 17 days post-reperfusion (Lee et al., 2008). However, this study did not observe a reduction in infarct volume in the rats that were exercised. In agreement with this latter study, our studies and some others evaluating the effect of post-stoke physical exercise on infarct volume in young male rats did not observe any reduction in infarct volume (Lee et al., 2008; Stradecki-Cohan et al., 2017). This suggests that other mechanisms are at play to improve cognitive recovery.

In addition to the putative beneficial effects of sex hormones described above, there are multiple other mechanisms that may be activated following exercise post-stroke. For example, an earlier study observed that forced physical exercise using rota-rod increased neurogenesis in subgranular zone of the dentate gyrus (Lee et al., 2008). Another study evaluating the effect of voluntary exercise post-stroke observed upregulation of proteins involved in neurogenesis (Mizutani et al., 2011). Thus, these studies suggest that enhanced neurogenesis in subgranular zone may be, in part, responsible for observed enhanced cognitive performance in aged male rats subjected to post-stroke exercise in our studies.

Increased angiogenesis induced by post-ischemic exercise is another potential mechanism. Ding et al. (2004) observed that a daily 30 min (15 m/min) treadmill exercise increased

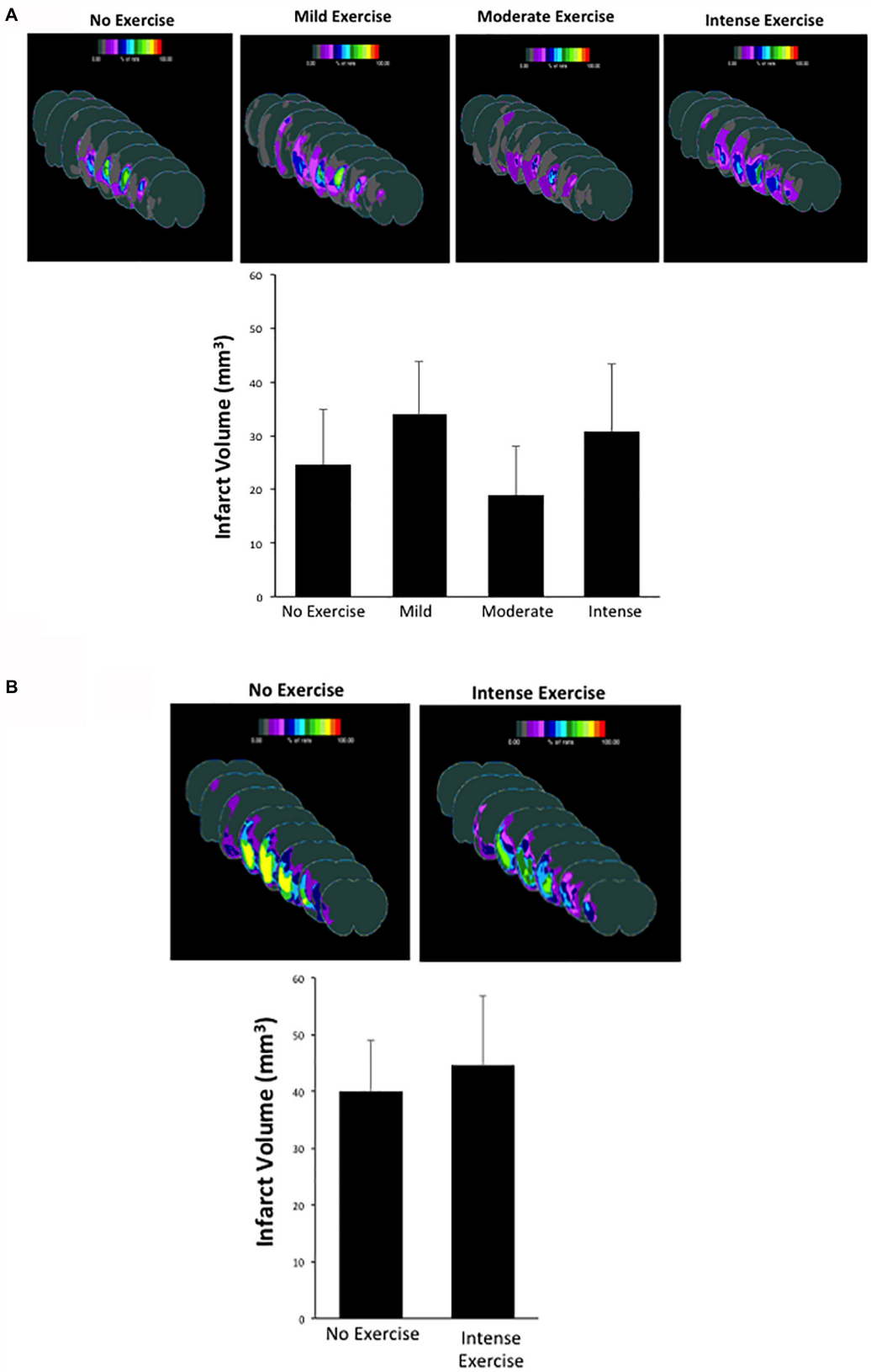

FIGURE 3 | (A,B) Infarct volume measurements observed in male and female rats that had undergone different exercise intensities following recovery from MCAO. There were no significant differences observed between groups (one way ANOVA, Bonferroni *post hoc* test).

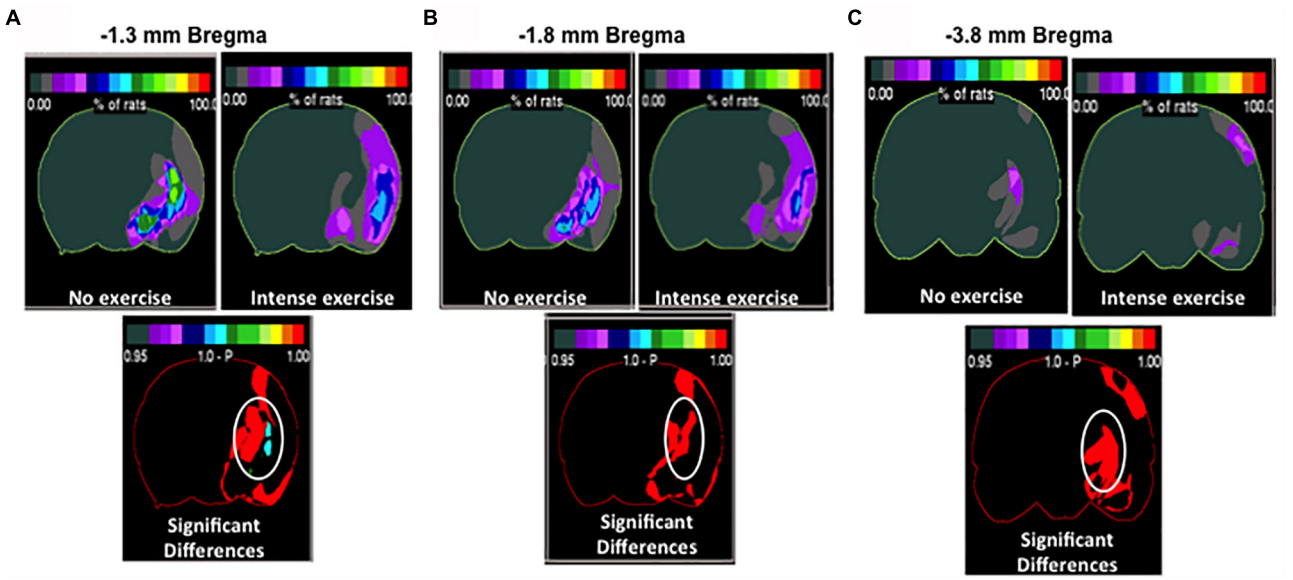

FIGURE 4 | Region specific infarct volume in aged male rats. Frequency heat map representation of infarct volume for each treatment group depicting location of region specific injury. Non-circled red is an increase in injury in the intense group. **(A)** Significant decrease in injury in the intense exercise group at –1.3 mm bregma was observed. There was an observed decrease in injury in the thalamus striatal injury and basal nucleus of Meynert. Non-circled red is an increase in injury in the intense group. **(B)** Highlighting –1.8 mm bregma showing a decrease in thalamic injury **(C)** –3.8 mm bregma showing a decrease in thalamic injury. Non-circled red is an increase in injury in the intense group.

levels of angiopoietin, mRNA levels of four VEGF isoforms, and microvessels density in both cortex and striatum. It is also possible that exercise-induced improvement in cognitive function may also be due to increased post-ischemic angiogenesis. As mention in the paragraph above, one of the mechanisms by which treadmill exercise confers protection against ischemic damage is via potential angiogenesis (Tang et al., 2018; Chen et al., 2019). An earlier study reported that in rodent brain, thalamic areas have higher capillary density compared to other brain areas (Xiong et al., 2017). It is plausible that more vascularized brain areas may thus have more beneficial impact of treadmill exercise compared to poorly vascularized brain area. More detailed studies are required to further confirm this hypothesis.

Although there were no differences in overall infarct volume between the different exercise groups, there were differences between specific areas in male rats. We observed significant reductions in infarct region found within the thalamus, and the cholinergic basal nucleus of Meynert in the intense exercise group as compared to no exercise group (**Figure 4**). In support of the current findings, a prior study observed functional disturbances and disruption of the cholinergic pathway between the frontal cortex and the basal nucleus of Meynert after middle cerebral artery occlusion in rats (Kataoka et al., 1991). Previous work has also indicated that injury to the thalamus can disrupt cognitive function (Savage et al., 2011). Injury to the basal nucleus of Meynert may play a role in mediating spatial memory function (Tian et al., 2004) and it has been proposed that damage to this nucleus is involved in the pathological mechanism of Alzheimer's disease (Grothe et al., 2012; Shu et al., 2019). Furthermore, in a rat model of Alzheimer's disease produced by lesion to cholinergic innervation, treadmill running delayed cognitive decline and prevented memory deficit (Hosseini et al., 2013). An increase in cortical cerebral blood flow via the activation of cholinergic neurons originating in the basal nucleus of Meynert can protect the ischemia-induced delayed death of cortical neurons by preventing a blood flow decrease in widespread cortices (Hotta et al., 2002). Therefore, it is possible that specific cell types within these regions are better protected from long term cell death occurring within the weeks following stroke. Further studies are warranted in order to determine if these regions are critical toward exercise dependent cognitive recovery.

SUMMARY

In this publication, we investigated the effects of aging and sex differences on different paradigms of forced treadmill exercise and their ability to modify cognitive recovery after stroke. We found that intense exercise was necessary in order to promote cognitive recovery in male animals (**Figure 2**). However, the same exercise intensity did not promote cognitive recovery in female animals (**Figure 2**). Future studies are being design to test what is the best PE intensity in young female rats. Neither of these paradigms decreased the total volume of the infarct (**Figures 3**, **4**). The efficacy of PE in lowering post-stroke cognitive impairment remains to be establish in animal models of stroke comorbidities. Overall, this paper elucidates an exercise paradigm that could

be used to promote recovery after stroke in aged male animals, however, future studies need to directly investigate specific exercise types and regimens as well as new strategies to promote the same recovery in aged female animals.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Care and Use of Laboratory Animals and approved by the University of Miami Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MP-P, KD, APR, CC, and MY conceived the scientific idea and designed the experiments. CC, MP-P, KD, and APR wrote the manuscript. RS, TR, and SK provided discussions on the project throughout and input in the writing of the manuscript. IS and CF performed the surgeries on rats to induce stroke. AAR, PP, and EP carried out the behavioral testing of rats. WZ and CD assisted with statistical analysis of images and data, respectively.

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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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Study on Effect of Striatal mGluR2/3 in Alleviating Motor Dysfunction in Rat PD Model Treated by Exercise Therapy

Ping Chen[1,2] *and Xiaodong Li*[1]*

[1]College of Sport Science, JiShou Univerity, JiShou, China, [2]College of Physical Education and Sports, Beijing Normal University, Beijing, China

Background: Exercise therapy has been widely applied in clinical rehabilitation as an important practical and side effect—free adjuvant therapy, with a significant effect in alleviating motor dysfunction of patients with Parkinson's disease (PD) or animal PD models. This study focuses on the effect of exercise therapy in reducing the concentration of extracellular glutamate (Glu) in the striatum in a rat PD model by upregulating the expression of group II metabotropic Glu receptor (mGluR2/3), so as to alleviate motor dysfunction in the rat PD model.

Methods: Neurotoxin 6-hydroxydopamine (6-OHDA) was injected into the right medial forebrain bundle (MFB) of the rats to establish the semi-lateral cerebral damage PD model. The sham-operated group was given an equal amount of normal saline at the same site and taken as the control group. The apomorphine (APO)-induced rotational behavior test combined with immunohistochemical staining with tyrosine hydroxylase (TH) in the substantia nigra (SNc) and striatum was performed to assess the reliability of the model. The exercise group was given treadmill exercise intervention for 4 weeks (11 m/min, 30 min/day, 5 days/week) 1 week after the operation. The open field test (OFT) was performed to assess the locomotor activity of the rats; the Western blot technique was used to detect SNc TH and striatal mGluR2/3 protein expressions; real-time polymerase chain reaction (RT-PCR) was applied to detect striatal mGluR2 and mGluR3 mRNA expressions; the microdialysis—high-performance liquid chromatography (HPLC) method was adopted to detect the concentration of extracellular Glu in striatal neurons.

Results: Compared with the control group, the number of rotations of each model group at the first week was significantly increased ($P < 0.01$); compared with the PD group, the number of rotations of the PD + exercise group at the third week and the fifth week was significantly decreased ($P < 0.05$, $P < 0.01$). Compared with the control group, the total movement distance, the total movement time, and the mean velocity of each model group at the first week were significantly reduced ($P < 0.05$); compared with the PD group, the total movement distance, the total movement time, and the mean velocity of the PD + exercise group at the third week and the fifth week were significantly increased

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***Correspondence:**
Xiaodong Li
802618@qq.com

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($P < 0.01$). Compared with the control group, the count of immunopositive cells and protein expression of SNc TH, and the content of immunopositive fiber terminals in the striatal TH of each model group significantly declined ($P < 0.01$). Compared with the PD group, the striatal mGluR2/3 protein expression of the PD + exercise group significantly rose ($P < 0.01$). Compared with the control group, the concentration of extracellular Glu in striatal neurons of each model group at the first week significantly grew ($P < 0.05$); compared with the PD group, the concentration of extracellular Glu in striatal neurons of the PD + exercise group at the third week and the fifth week was significantly decreased ($P < 0.01$); compared with the PD + exercise group, the concentration of extracellular Glu in striatal neurons of the group injected with mGluR2/3 antagonist (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA) into the striatum at the third week and the fifth week was significantly increased ($P < 0.05$, $P < 0.01$). Compared with the control group, the striatal mGluR2/3 protein expression of the PD group was significantly downregulated ($P < 0.01$); compared with the PD group, the striatal mGluR2/3 protein expression of the PD + exercise group was significantly upregulated ($P < 0.05$); compared with the control group, the striatal mGluR3 mRNA expression of the PD group was significantly downregulated ($P < 0.01$); compared with the PD group, the striatal mGluR3 mRNA expression of the PD + exercise group was significantly upregulated ($P < 0.01$); 6-OHDA damage and exercise intervention had no significant effect on the striatal mGluR2 mRNA expression ($P > 0.05$). Compared with the PD + exercise group, the total movement distance, the total movement time, and the mean velocity of the PD + exercise + APICA group were significantly decreased ($P < 0.05$); compared with the PD group, the PD + exercise + APICA group had no significant change in the total movement distance, the total movement time, and the mean velocity ($P > 0.05$).

Conclusion: These data collectively demonstrate that the mGluR2/3-mediated glutamatergic transmission in the striatum is sensitive to dopamine (DA) depletion and may serve as a target of exercise intervention for mediating the therapeutic effect of exercise intervention in a rat model of PD.

Keywords: 6-OHDA, exercise, rat Parkinson's disease model, striatum, glutamate, mGluR2/3, motor dysfunction

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases in the world and seriously affects the quality of life and the health of middle-aged and elderly people (Poewe et al., 2017; Haertner et al., 2018; Masilamoni and Smith, 2018; Oliveira de Carvalho et al., 2018; Stoessel et al., 2018). Currently, the pathogenesis of PD is still unclear (Carnwath et al., 2018), with a lack of an ideal therapeutic regimen in the clinic. According to most researchers, the primary pathologic changes of PD are that the degeneration and loss of dopaminergic neurons in the midbrain substantia nigra (SNc) cause the reduction of the dopamine (DA) release in the SNc—striatum pathway, the decrease of the direct pathway activity and the increase of the indirect pathway activity in the basal ganglia (BG), and the over-inhibition of thalamic and cortical neurons, which therefore lead to a clinical syndrome characterized by motor dysfunctions, such as bradykinesia, muscular rigidity, static tremor, gait disturbance, and postural instability (Ali and Morris, 2015; Hu et al., 2018; Stephano et al., 2018; Chen et al., 2019). Therefore, the main target for the treatment of PD is to deactivate the indirect pathway by enhancing dopaminergic neurotransmission or reducing glutamatergic neurotransmission.

Glutamate (Glu) is one of the primary excitatory neurotransmitters in the central nervous system. It plays a central role in the fundamental functions of the brain, including synaptic plasticity (critical for learning and memory), and the formation of neural networks during the development and repair of the central nervous system (McEntee and Crook, 1993; Meldrum, 2000). Due to the role of Glu in the neural circuits of the BG, it is also essential in motor control (Blandini et al., 1996). However, in some cases, over-continuous activation of Glu can damage nerve tissue and involve the occurrence of a variety of brain diseases (Blandini, 2010). According to the findings in recent years, in PD patients or neurotoxin-induced

PD model animals, the depletion of SNc–striatum DA could cause the excessive activation of the cortex–striatum Glu pathway, release plenty of presynaptic Glu, and activate the striopallidal GABAergic pathway (the indirect pathway is overactive; Klockgether and Turski, 1989; Gerfen, 1992; Blandini et al., 2000; Wichmann and Delong, 2007). Therefore, blocking the excessive release of presynaptic Glu of the cortex–striatum pathway or inhibiting Glu's effects could reduce glutamatergic transmission as well as indirect pathway activity. Glu exerts its biological effects through mediation of its receptors. Glu receptors are classified into iontropic Glu receptors (iGluRs) and metabotropic Glu receptors (mGluRs; Lau and Tymianski, 2010; Litim et al., 2017; Jenner and Caccia, 2019). In recent years, extensive studies have focused on the effect of iGluRs in the occurrence and development of PD and put forward that the excitotoxicity of Glu may be one of the important mechanisms in the occurrence and development of PD (DeLong and Wichmann, 2015; Van Laar et al., 2015). Many studies have indicated that although iGluR antagonist has an anti-PD effect, it is still restricted because the receptor is not specifically distributed in the central nervous system, and nonselective iGluR antagonist may have significant side effects, like cognitive dysfunction and psychotomimetic symptoms, in clinical experiments (Dell'anno et al., 2013; Masilamoni and Smith, 2018). Therefore, researchers have turned to mGluRs and found that mGluR2/3 may be an important target for the treatment of PD (Nicolletti et al., 2011). Group II mGluR is a cortex–striatum autoreceptor located at the presynaptic terminal, and its activation can reduce the cortex–striatum Glu release at the presynaptic terminal. Currently, mGluR2/3 agonist has been partially applied in clinical treatment, with a significant efficacy (Litim et al., 2017). According to the findings of an epidemiological survey, exercise/body movement can reduce the onset risk of PD (Lauzé et al., 2016); clinical and basic studies have verified that different forms of exercise/body movement can alleviate symptoms or delay the development of symptoms of patients with PD or animal PD models (Cheng et al., 2016; Sheibani et al., 2017). Therefore, it is inferred in this study that exercise intervention may have an effect in alleviating motor dysfunction in the rat PD model by upregulating the striatal mGluR2/3 protein expression, reducing the Glu release at the presynaptic terminal, and then decreasing the activity of the indirect pathway. In this study, *in vivo* microdialysis–high-performance liquid chromatography (HPLC), real-time polymerase chain reaction (RT-PCR), Western blot, and other molecular biological techniques were adopted to explore the effect of exercise intervention on the concentration of extracellular Glu in striatal neurons, the striatal mGluR2/3 mRNA expression, and the striatal mGluR2/3 protein expression in the rat PD model; the intervention with mGluR2/3 antagonist further confirmed the significant regulatory effect of mGluR2/3 on the concentration of extracellular Glu in striatal neurons and the motor function of the rats and provided experimental evidence for the hypothesis that exercise may alleviate the excitotoxicity caused by excessive activation of the cortex–striatum Glu at the synapse by upregulating mGluR2/3 and reducing the concentration of extracellular Glu in striatal neurons.

MATERIALS AND METHODS

Experimental Animals

Healthy clean-grade male SD rats weighing 240 ± 10 g (6 weeks old) were provided by Beijing HFK Bioscience Company Limited [Beijing, China; production license no. SCXK (BJ) 2009-0007]. The rats were fed in separate cages (three to four rats per cage), and kept on a 12:12 h light-dark cycle at a room temperature of 20–25°C with free access to food and water. During the experiment, the rats were given humanitarian care in the 3R principle for experimental animals. Before the formal experiment, they were enrolled in a 7-day adaptive exercise and forced treadmill exercise, and those incapable of finishing the preset treadmill exercise were excluded. The animal study was reviewed and approved by the experimental animal ethics committee, School of Physical Education and Sports, Beijing Normal University (IACUS-BNU-NKLCNL2016-02). The experimental design flowchart is as follows (**Figure 1**).

Modeling and Assessment

The rats were fasted for 24 h with free access to water, and then intraperitoneally injected with 10% chloral hydrate (0.35 ml/100 g) for deep anesthesia, fixed in a prone position on a rat digital stereotaxic instrument (RWD, Shenzhen, China), and kept warm at 37°C with a thermostatic heating pad. At 30 min before injection with 6-hydroxydopamine (6-OHDA), they were intraperitoneally injected with desipramine (25 mg/kg) for protection of norepinephrine serotonergic neurons. Rat hair at the calvaria was shaved to expose the scalp; the skin of the operative area was disinfected with polyninylpyrrolidone; the scalp and periosteum were cut open with surgical scissors along the central line of the skull; and the bone surface was wiped with cotton balls dipped in hydrogen peroxide (H_2O_2) to fully expose the anterior and posterior fontanelles, and the anterior and posterior fontanelles were kept at the same horizontal level. Paxinos and Watson's stereotaxic coordinates (Paxinos and Watson, 1997) of the right medial forebrain bundle (MFB) were anterior fontanel (AP): −4.3 mm, right (R): 1.5 mm, and deep (D): 7.6–7.8 mm. Based on the above coordinates, a skull hole was drilled by a dental drill and then injected with 4 μl 6-OHDA (2 μg/μl, including 0.02% ascorbic acid and 0.9% normal saline with an injection velocity of 0.5 μl/min). The sham-operated group was injected with 4 μl 0.9% normal saline (including 0.02% ascorbic acid) at the same site by the same method. After injection, the needle was retained for 10 min before slowly retreating (with the velocity at 1 mm/min), and then the skull hole was filled with biological silica gel.

After injection with 6-OHDA or normal saline, the microdialysis probe cannula and the mGluR2/3 antagonist [(RS)-1-amino-5-phosphonoindan-1-carboxylic acid, APICA] delivery catheter were implanted at the right striatum (AP: +1 mm, R: 2.5 mm, D: 3.5 mm; AP: −1.0 mm, R: +1.6 mm, D: 4.0 mm; Jia et al., 2017) and fixed at the skull surface by three to five small stainless steel screws and dental cement (**Figure 2**). After the operation, the rats were fed in separate

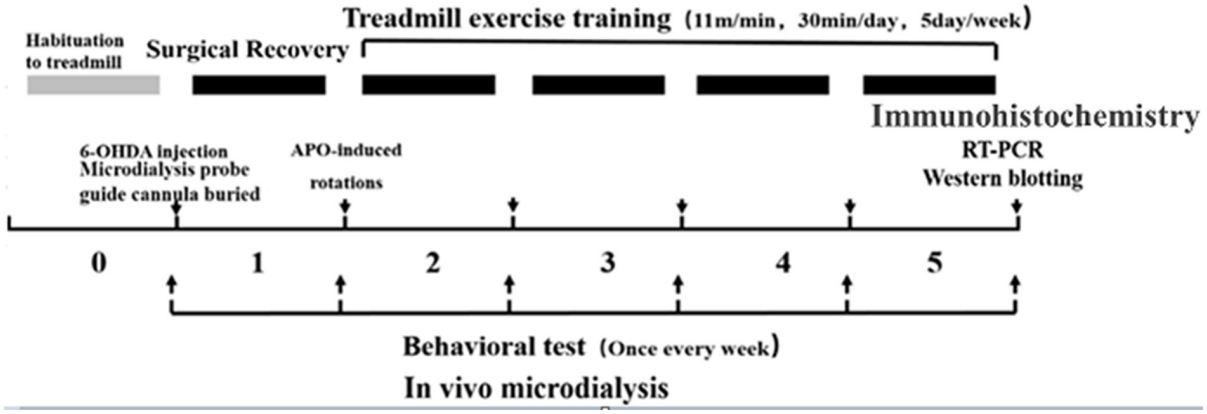

FIGURE 1 | Experimental design flowchart.

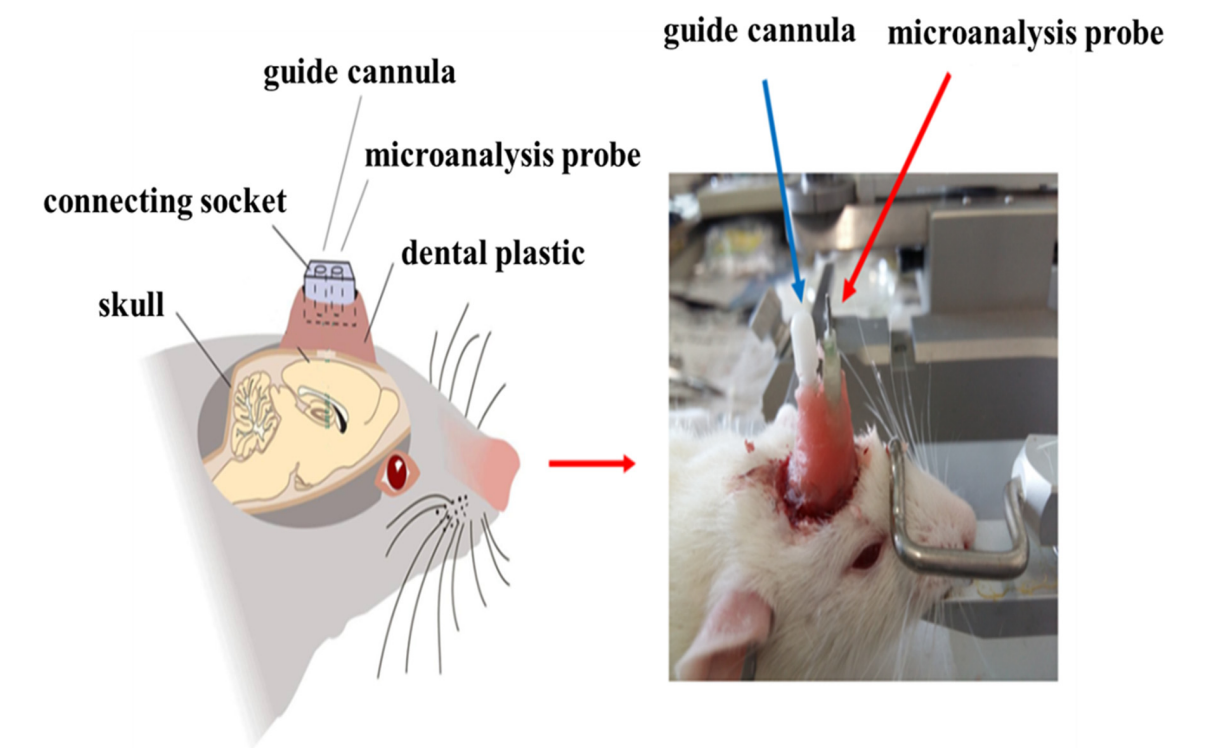

FIGURE 2 | Imbedding of microdialysis cannula and drug delivery catheter.

cages and intraperitoneally injected with penicillin to prevent postoperative injection for successively 3 days.

At the seventh day after the operation, the apomorphine (APO)-induced rotational behavior test was performed to verify the successful establishment of the 6-OHDA semi-lateral cerebral damage model. The experiment was performed in a tranquil environment. APO solution (0.1 mg/100 g·wt·d) was injected subcutaneously into the neck of the rats, and the number of rotations (in which the rats rotated head to tail with the left posterior limb as the axis) for 30 min was recorded. In this study, the criterion for successful establishment of the rat PD model was the net number of rotations (namely, the number of counterclockwise rotations minus the number of clockwise turns rotations) >100 r/30 min.

Grouping

The model rats were randomly divided into three groups: 6-OHDA sedentary group (PD, n = 12), 6-OHDA + exercise

group (PD + Ex, n = 12), and 6-OHDA + exercise + mGluR2/3 antagonist group (PD + Ex + APICA, n = 12). The sham-operated group was taken as the sedentary control group (control, n = 12).

Exercise Intervention and Dosage Regimen

At the first week after the operation, the exercise regimen designed by Tajiri et al. (2010) was adopted for treadmill exercise intervention in the PD + exercise group and the PD + exercise + APICA group. The exercise regimen lasted for 4 weeks (11 m/min, 30 min/day, 5 days/week, rest on Saturday and Sunday). The treadmill exercise intervention time was 16:00–18:00 in the afternoon of each exercise day. At 20 min before each exercise, the PD + exercise + APICA group was injected with mGluR2/3 antagonist APICA inside the striatum by a microinjection pump, with injection volume of 1 μl. The control group and the PD group were injected with the same volume of normal saline within the same period and put in the treadmill but in a sedentary state without treadmill exercise.

Assessment of Rat Locomotor Activity

The open field test (OFT) was performed to assess the locomotor activity of the rats (Sáenz et al., 2006). The OFT chamber (origin: Spain, brand: Panlab, supplier: RWD, Shenzhen, China) was 40 cm high and 100 cm wide and long, with gray non-transparent walls and a black bottom, and placed in a non-background anechoic chamber with a light intensity of 20 lux. A digital video camera [SONY (China) Company Limited, Shenzhen, China] was put at 80 cm above the OFT device and could cover the entire open field. After 60 min of the rats adapting to the experimental room, the rats were placed in the OFT chamber, and then after 0.5 min of being adapted to the open field, formal test recording was done. The Smart 3.0 software (origin: Spain; brand: Panlab; supplier: RWD, Shenzhen, China) was used to record the locomotor activity behaviors of the rats for 30 min, and the environment was kept tranquil during the whole test. After the test, built-in software Smart 3.0 was used to analyze the video of each rat (**Figure 3**).

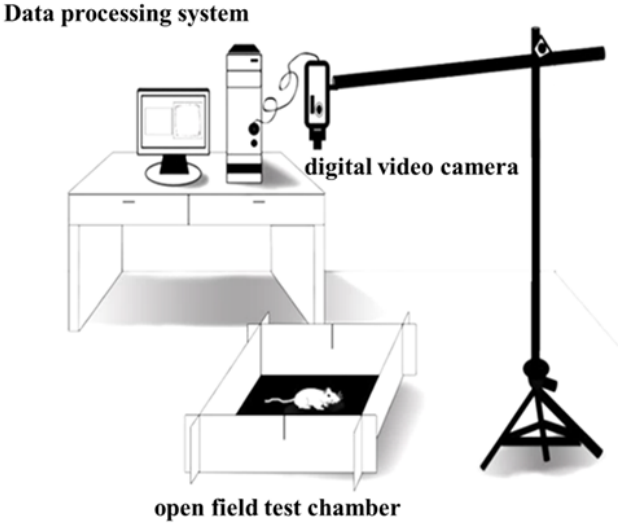

FIGURE 3 | Rat locomotor activity detection mode.

Striatal Microdialysis Sample Liquid Collection and Glu Concentration Determination

Microdialysis Cannula and Drug Delivery Catheter Imbedding Operation and Sample Collection

Before the collection of microdialysis samples, the guide probe core was removed, and then the probe was slowly inserted into the cannula and fixed. The rats were placed in the waking activity device. The probe input end was connected to the microinjection pump, and the output end was connected to the frozen collector. The microsyringe pump was filled with artificial cerebrospinal fluid, which was continuously perfused with a velocity of 0.2 μl/min. At 30 min after perfusion to achieve the balance state, the frozen collector began collecting microdialysis liquid samples with a velocity of 15 min per tube, one time per week, for four successive weeks. After collection, the samples were preserved at −80°C in a refrigerator (**Figure 4**).

Rat Cerebral Histological Location

After the microdialysis sample collection, the rats were anesthetized with 10% chloral hydrate (0.35 ml/100 g); the thoracic cavity was cut open to expose the heart, which was perfused and fixed with 4% paraformaldehyde solution; and cerebral tissues were stripped and sunk in 30% sucrose-buffered paraformaldehyde solution overnight. The cerebral tissues were prepared into serial coronary frozen slices (40 μm), which were Nissl-stained to verify the locations of the microdialysis cannula and drug delivery catheter, and compared with cerebral stereotaxic coordinates, so as to eliminate the dialysate samples with microdialysis probe cannula and drug delivery catheter imbedding locations outside in the striatum.

Glu Precolumn Derivatization Fluorescence Detection Method

The precolumn derivatization fluorescence detection method was used to determine the striatal Glu concentration. Glu's chromatographic mobile phase was composed of 0.1 mol/L potassium dihydrogen phosphate solution (pH downregulated to 6.6) and pure methanol (40% for isorheic elution). Before use, it was filtered with 0.22 μm organic filter membranes and degassed through ultrasonic vibration. The flow rate was set at 1 ml/min. The SHIMADZU ODS-SP (4.6 × 150 mm, 5 μm) chromatographic column was adopted, with the column oven temperature at 25°C, the excitation wavelength at 357 nm and the emission wavelength at 455 nm. According to the precolumn derivatization method, 13.5 mg ortho-phthalaldehyde was first weighed and dissolved in 250 μl pure methanol; then, 25 ml prepared boric acid solution (0.4 mol/L) was added and mixed evenly; and finally, 100 μl β-mercaptoethanol was drop-wise added and protected from light at 4°C. Subsequently, 0.53 g Na_2CO_3 was weighed, fully dissolved in 100 ml ultrapure water, and prepared into

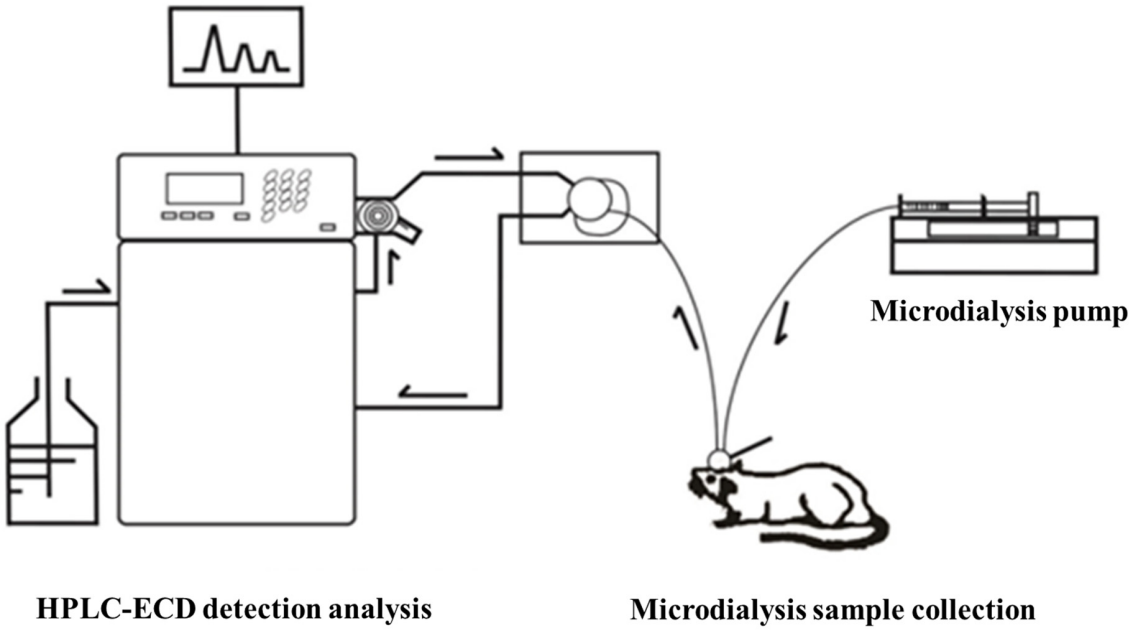

FIGURE 4 | Microdialysis sample liquid collection of rat striatum in the waking state.

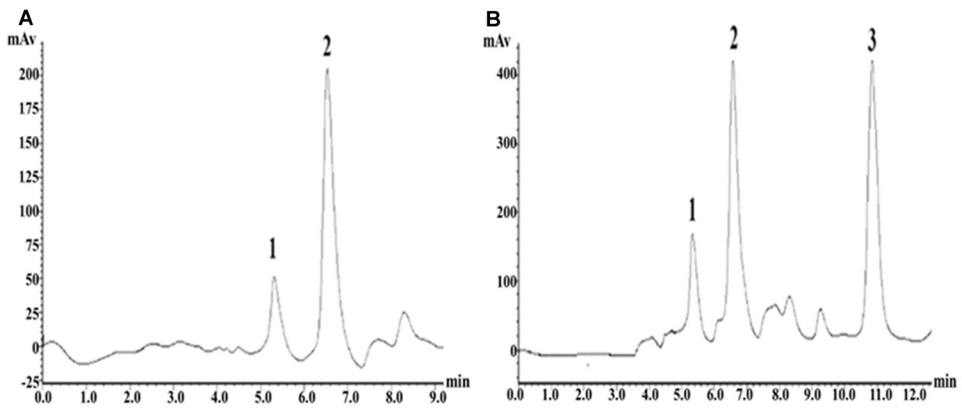

FIGURE 5 | High-performance liquid chromatography (HPLC) fingerprints of Glu standard samples and striatal microdialysis liquid samples. Note: **(A)** HPLC fingerprints of glutamate (Glu) standard sample, in which 2 represents Glu. **(B)** Rat striatal microdialysis–HPLC, in which 2 represents Glu, and 1 and 3 are unknown.

Na_2CO_3 buffer (0.05 mol/L). Glu standard substances were accurately weighed and prepared into standard solutions (10, 1, 0.1, 0.01, 0.001 μmol/L), or the mother solution was first prepared and then diluted in sequence. Twenty microliters of dialysate/standard solution was weighed, added with 10 μl derivating agent and 10 μl Na_2CO_3 buffer (0.05 mol/L); they were fully mixed and put aside for 30 s; 20 μl was extracted for sampling. The concentration-peak area standard curve was drawn in LC-Solution software based on the corresponding peak areas of the five concentration standard samples. With the peak area as vertical coordinate Y and the standard sample concentration as horizontal ordinate X, the standard curve was $Y = 1179939.70X - 19522.40$ ($r^2 = 1.0000$). The chromatographic peaks of the samples were qualitatively analyzed according to the Glu peak retention time; the area of the chromatographic peak with the same retention time (±0.1) as the standard sample was obtained by LC-Solution software, and the sample concentration was quantitatively based on the standard curve (**Figure 5**).

TH and MGluR2/3 Detection

Immunohistochemistry

The immunohistochemical technique was adopted to detect the count of tyrosine hydroxylase (TH)—immunopositive cells of the SNc and the content of TH-immunopositive fiber terminals in the striatum. After the behavior test at the last week, the

rats were fasted for 24 h, then intraperitoneally injected with 10% chloral hydrate (0.35 ml/100 g) for anesthesia, and perfused with 0.9% normal saline (250 ml) and 4°C 4% PFA solution (250 ml) *via* the ventriculus sinister through the ascending aortic cannulation. After the perfusion, the whole cerebral tissues were taken out and fixed in paraformaldehyde solution for 24 h. The cerebral tissues were dehydrated, trimmed and embedded after being taken out. When being sliced, the coronal plane of the cerebral tissues was first trimmed. Striatum and SNc locations were determined by reference to Paxinos and Watson's (1997) stereotaxic coordinates. Serial coronary slices were made around each determined site, and one out of every other three slices was selected, with a thickness of 5 μm. The cerebral slices were rinsed with 0.01 M PBS (pH 7.4) and put in 0.3% Triton X-100 PBS at room temperature for 30 min for cell rupture. Then, they were incubated with 3% H_2O_2 and rinsed with PBS. The cerebral slices were transferred to PBS of 5% normal goat serum (haoranbio, China) for 1 h incubation at room temperature and then incubated with rat anti-TH monoclonal antibodies (1:3,000, Sigma, USA) overnight. After being rinsed with PBS three times, the cerebral slices were incubated with biotinylated rabbit antibodies (Millipore, USA) for 1 h at room temperature, then incubated with avidin-biotin-peroxidase compound (ABC-Elitekit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature, rinsed with PBS three times, and stained with DAB solution for 10–20 s. An Olympus-DP72 microscope (Olympus, Japan) was used for microphotography, and Image-Pro Plus 6.0 was adopted for statistics and analysis for the count of immunopositive cells of SNc TH and the content of immunopositive fiber terminals in the striatal TH (mean optical density), so as to determine the damage of dopaminergic neurons.

Western Blot

Western blot technique was adopted to detect SNc TH and striatal mGluR2/3 protein expressions. In the last week, at 24 h after the behavior test, the rats were intraperitoneally injected with 10% chloral hydrate (0.35 ml/100 g) for deep anesthesia and decapitated; then, the cerebral tissues were taken out. The right striatum and the ventral mesencephalon were quickly stripped. After the protein content was determined by the bicinchoninic acid (BCA) method, the cerebral tissues were added with 5-fold SDS, boiled in water for 5 min, and then cooled and preserved in a refrigerator at −80°C. Thirty-microgram protein samples were taken for electrophoretic separation, placed on polyvinylidene fluoride (PVDF) membrane and then in plastic wrap, added with confining liquid, and slowly shaken at room temperature for 90 min. The membrane was sealed in 5% nonfat milk dissolved in Tris-buffered saline and Tween20 (TBST); added with rat primary antibodies TH (Abcam, UK) or rabbit primary antibodies mGluR2/3 (Millipore, USA), incubated at 4°C for 24 h, and rinsed with PBS; and then added with sheep anti-rat and sheep anti-rabbit secondary antibodies (Univ, China), placed on a shaker, incubated at room temperature for 90 min, and rinsed. After reaction at room temperature for 1 h, the membrane was added with chemical fluorescent liquid, followed by exposure and development with X-ray film. With β-actin as the internal control, ImageJ image analysis software was used to analyze the images. The relative protein content was represented by the ratio of the integral optical density (IOD) of each band to the IOD value of its corresponding β-actin.

Real-Time Polymerase Chain Reaction

By reference to the literature of Zhang et al. (2009), SYBR Green I RT-PCR was used to detect the changes in the striatal mGluR2 and mGluR3 mRNA transcription levels. According to gene cDNA sequencing of *Rattus* listed on GenBank, Primer software was used to design primers. As for mGluR2, the upstream primer was 5′-TGGCACAGGCAAGGAGACAG-3′, the downstream primer was 5′-GCGATGAGGAGCACATTG TAGG-3′, and the amplified product size was 111 bp. As for mGluR3, the upstream primer was 5′-GAAGCCGAGTATAT GTGTCCTG ATG-3′, the downstream primer was 5′-CACT GCTGTATGAACCACCAATGA-3′, and the amplified product size was 94 bp. As for the internal control GAPDH, the upstream primer was 5′-TGGAGTCTACTGGCGTCTT-3′, the downstream primer was 5′-TGTCATATTTCTCGTGG TTCA-3′, and the amplified product size was 138 bp.

Statistics and Analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was adopted for statistics and analysis for all of the data. The results are represented by mean ± standard deviation ($\bar{x} \pm$ SD). Sigmaplot 12.5 software was used for mapping. The means of groups were compared by one-way analysis of variance (ANOVA). Inter-group mean differences were compared by LSD test, while intra-group mean differences were compared by repeated-measure two-way ANOVA. The differences were considered statistically significant when the *P*-value was less than 0.05.

RESULTS

Effect of Exercise Intervention in Alleviating Motor Dysfunction in Rat PD Model

According to the results of the rotational behavior experiment, compared with the control group, the number of rotations of the PD group and the PD + exercise group at the first week was significantly increased, with statistically significant differences ($P < 0.01$); compared with the PD group, the number of rotations of the PD + exercise group at the third week was decreased, with statistically significant differences ($P < 0.05$); and the differences were very statistically significant at the fifth week ($P < 0.01$, **Figure 6**).

According to the results of the OFT, compared with the control group, the total movement distance, the total movement time, and the mean velocity of the PD group and the PD + exercise group at the first week were reduced, with statistically significant differences ($P < 0.05$); compared with the PD group, the total movement distance, the total movement time, and the mean velocity of the PD + exercise group were increased, with statistically significant differences ($P < 0.05$); and the differences were very statistically significant at the fifth week ($P < 0.01$, **Figures 6B–D**).

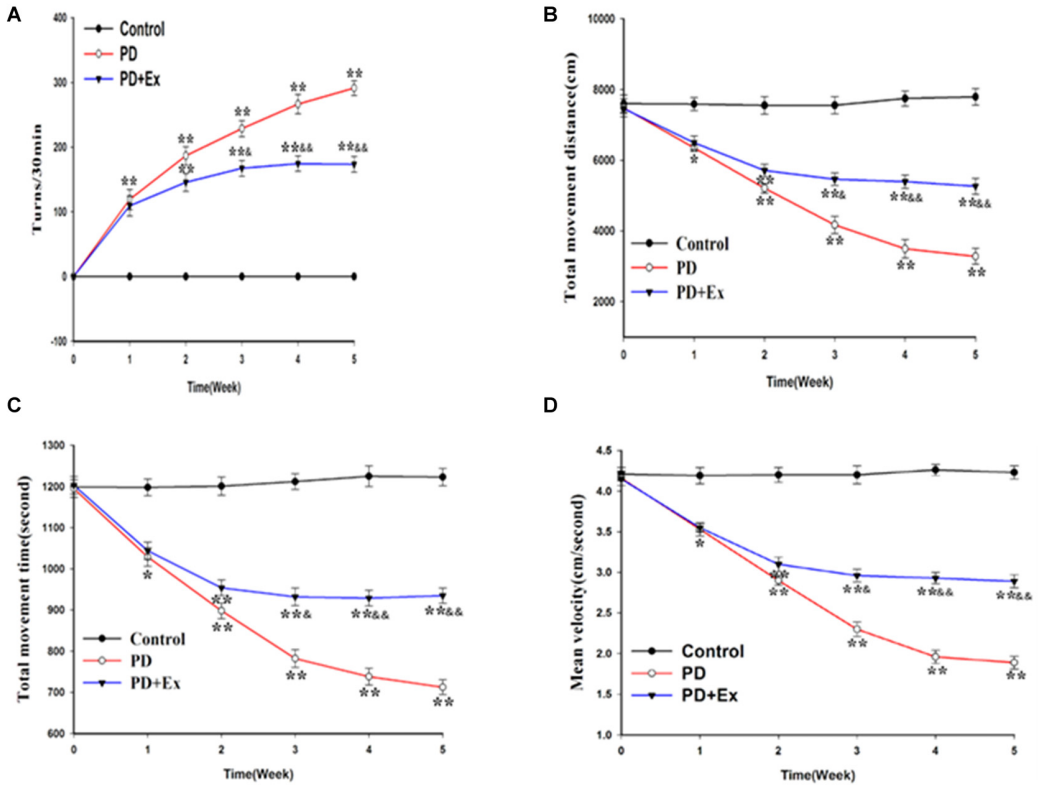

FIGURE 6 | Effect of exercise intervention on motor dysfunction rat Parkinson's disease (PD) model. **(A)** Apomorphine (APO)-induced rotational behavior of rats. **(B)** Changes in total movement distance of each group. **(C)** Changes in total movement time of each group. **(D)** Changes in mean velocity of each group. Compared with the control group, $^{*}P < 0.05$ and $^{**}P < 0.01$; compared with the PD group, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$.

No Effect of Exercise Intervention in Preventing Rat PD Model From Losing Dopaminergic Neurons

Compared with the control group, the count of immunopositive cells and protein expression of SNc TH, and the content of immunopositive fiber terminals in the striatal TH of the PD group declined, with statistically significant differences ($P < 0.01$); compared with the PD group, the count of immunopositive cells and protein expression of SNc TH, and the content of immunopositive fiber terminals in the striatal TH of the PD + exercise group had no significant change, with no statistically significant difference ($P > 0.05$, **Figure 7**).

Effect of Exercise Intervention in Reducing Concentration of Extracellular Glu in Striatal Neurons in Rat PD Model

Compared with the control group, the concentration of extracellular Glu in striatal neurons in the rat PD model at the third week and the fifth week significantly increased, with statistically significant differences ($P < 0.01$); compared with the PD group, the concentration of extracellular Glu in striatal neurons of the PD + exercise group at the third week and the fifth week significantly decreased, with statistically significant differences ($P < 0.05$, $P < 0.01$); compared with the PD + exercise group, the extracellular Glu in striatal neurons of the PD + exercise + APICA group at the third week and the fifth week was significantly increased, with statistically significant differences ($P < 0.05$, $P < 0.01$). Besides, the changes in the concentration of extracellular Glu in striatal neurons were negatively correlated with the changes in the locomotor activity of rats; with the increase of the exercise intervention time, the correlation was more significant at the fifth week ($P < 0.05$) than at the third week ($P < 0.01$, **Figure 8**).

Effect of Exercise Intervention in Re-regulating Striatal mGluR2/3 Expression in Rat PD Model

At the mRNA level, compared with the control group, the striatal mGluR3 mRNA expression level of the PD group significantly declined, with statistically significant differences ($P < 0.01$); compared with the PD group, the striatal mGluR3 mRNA expression level of the PD + exercise group was significantly increased, with statistically significant differences ($P < 0.01$). Both 6-OHDA damage

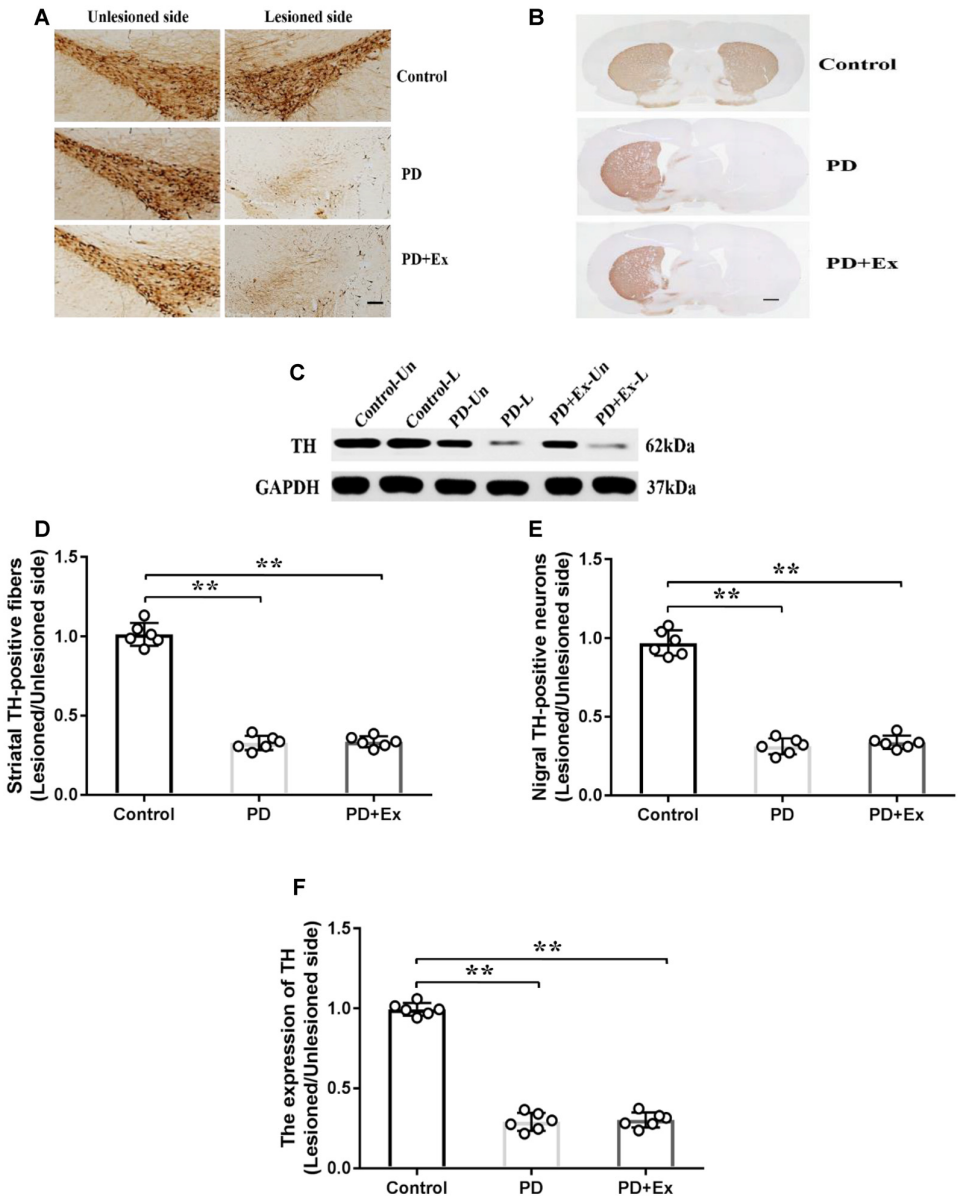

FIGURE 7 | Effect of exercise intervention on substantia nigra (SNc)–striatum dopaminergic system in rat PD model. **(A)** Damaged side/undamaged side SNc tyrosine hydroxylase (TH)–immunopositive cell staining (scale bar = 50 μm). **(B)** Damaged side/undamaged side striatal TH-immunopositive fiber staining (scale bar = 50 μm). **(C)** Western blot of ventral mesencephalon TH protein expression. **(D)** Damaged side/undamaged side SNc TH-immunopositive cell optical density ratio. **(E)** Damaged side/undamaged side striatal TH-immunopositive fiber terminal content ratio. **(F)** Damaged side/undamaged side ventral mesencephalon TH protein expression ratio. Compared with the control group, $^{**}P < 0.01$.

and exercise intervention had no significant effect on the striatal mGluR2 mRNA expression level, with no statistically significant difference ($P > 0.01$). At the protein level, compared with the control group, the striatal mGluR2/3 protein expression of the PD group was decreased, with statistically significant differences ($P < 0.01$); compared with the PD group, the striatal mGluR2/3 protein expression of the PD + exercise group was increased, with statistically significant differences ($P < 0.05$, **Figure 9**).

Effect of mGluR2/3 Antagonist in Preventing Exercise Intervention–Mediated Locomotor Activity Improvement in Rat PD Model

Compared with the PD + exercise group, the total movement distance, the total movement time, and the mean velocity of the PD + exercise + APICA group declined, with statistically significant differences ($P < 0.05$); compared with the PD group,

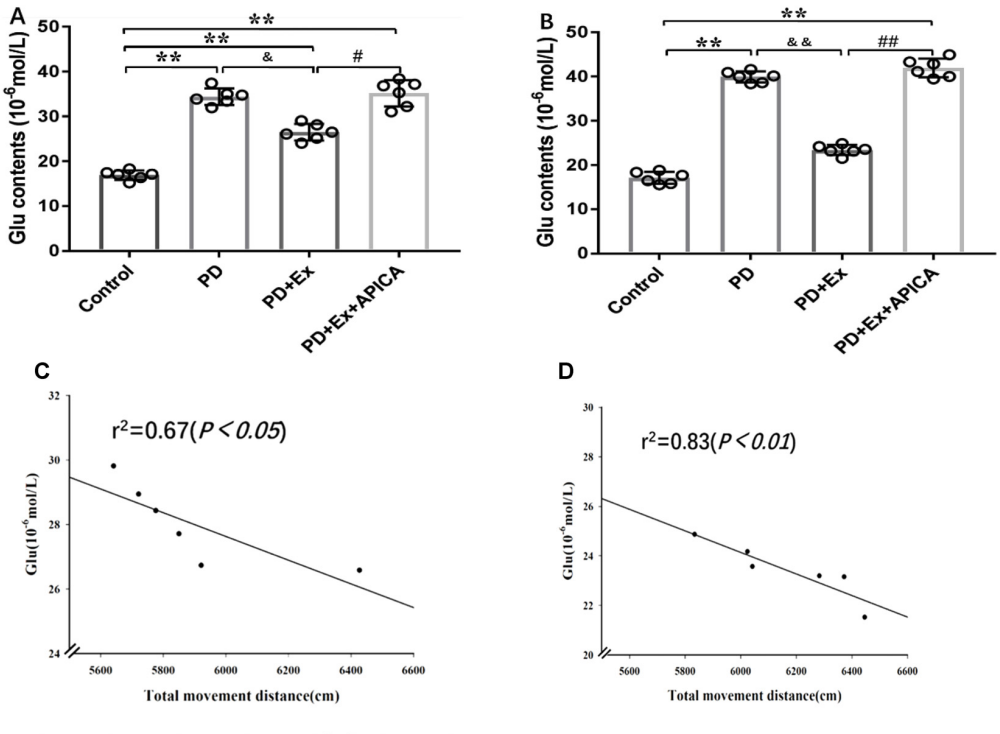

FIGURE 8 | Effect of exercise intervention on concentration of extracellular Glu in striatal neurons in rat PD model. **(A)** Effect of 2-week exercise intervention on concentration of extracellular Glu in striatal neurons. **(B)** Effect of 4-week exercise intervention on concentration of extracellular Glu in striatal neurons. **(C)** Effect of 2-week exercise intervention on correlation between concentration of extracellular Glu in striatal neurons and locomotor activity in rat PD model. **(D)** Effect of 4-week exercise intervention on correlation between concentration of extracellular Glu in striatal neurons and locomotor activity in rat PD model. Compared with the control group, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; compared with the PD group, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$.

the PD + exercise + APICA group had no significant change in the total movement distance, the total movement time, and the mean velocity, with no statistically significant difference ($P > 0.05$, **Figure 10**).

Effect of Exercise Intervention on Glu Release by Regulating mGluR2/3

Through reverse microdialysis, antagonist APICA was perfused to the striatum of normal rats to prevent mGluR2/3 and significantly increase the extracellular Glu content in the striatum. The treadmill exercise intervention could significantly reduce the extracellular Glu content in the striatum. After exercise, APICA was re-perfused to the striatum to increase the extracellular Glu content. However, the perfusion of mGluR2/3 agonist APDC could decrease the extracellular Glu content in the striatum (**Figure 11**).

DISCUSSION

This study explores the effect of the mGluR2/3-medicated glutamatergic system on dopaminergic neuron damage and exercise intervention's efficacy in alleviating motor dysfunction in the rat PD model. According to the findings, exercise intervention alleviates motor dysfunction in the rat PD model, upregulates the striatal mGluR2/3 expression level, and reduces the concentration of extracellular Glu in striatal neurons. Besides, mGluR2/3 antagonist APICA prevents the effect of exercise intervention in alleviating motor dysfunction in the rat PD model. These findings verify that the effect of exercise intervention in alleviating motor dysfunction in the rat PD model is partially achieved by the mGluR2/3 dependency mechanism.

Effect of Exercise Intervention in Alleviating Motor Dysfunction in Rat PD Model by Reducing Concentration of Extracellular Glu in Striatal Neurons

According to the findings of an epidemiological survey, people who are engaged in regular physical exercise in early age have a much lower incidence of PD than the general population (Sasco et al., 1992; Logroscino et al., 2006; Petzinger et al., 2015; Marica et al., 2018; Müller and Myers, 2018). Based on clinical observation, different forms of body movements (like treadmill exercise, resistive exercise, balance exercise, stretching exercise, deep breathing exercise, shadowboxing, dancing, and boxing) have a certain active effect in alleviating motor dysfunctions (such as bradykinesia, muscle rigidity, static tremor, titubation, and postural instability) of PD patients (Dashtipour et al., 2015; Hou et al., 2017; Giardini et al., 2018; Hirsch et al., 2018; Rawson et al., 2019). On the basis of the findings of animal PD model

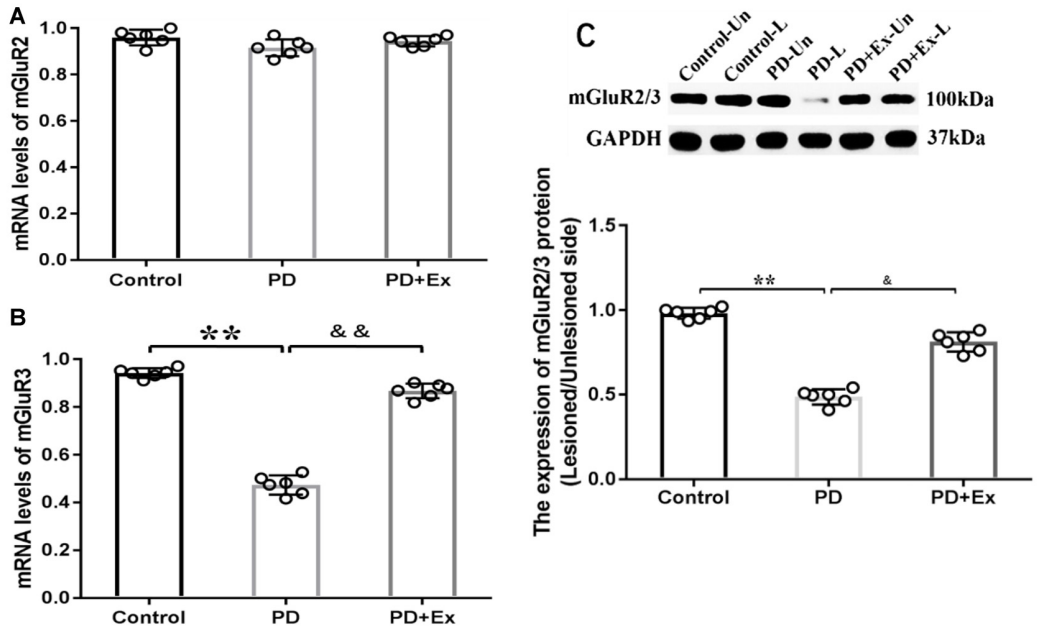

FIGURE 9 | Effect of exercise intervention on striatal metabotropic Glu receptor (mGluR2/3) mRNA and protein expressions in rat PD model. **(A)** Effect of exercise intervention on striatal mGluR2 mRNA expression level in rat PD model. **(B)** Effect of exercise intervention on striatal mGluR3 mRNA expression level in rat PD model. **(C)** Effect of exercise intervention striatal mGluR2/3 protein expression level in rat PD model. Compared with the control group, $^{**}P < 0.01$; compared with the PD group, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$.

studies, different forms of exercise intervention have an active effect in alleviating motor dysfunction in animal PD models (Hsueh et al., 2018; Jang et al., 2018; Ohno et al., 2018; Speck et al., 2019). Yoon and Lee (2014) reported that 4-week balance and gait exercises can significantly alleviate motor dysfunction in an MPTP-induced rat PD model, which is manifested as a significant increase in the rat movement time in the OFT case and a significant reduction in the pole-climbing delay time; Viana et al. (2017) found that long-term moderate-intensity treadmill endurance exercise can significantly alleviate motor dysfunction in the mouse PD model, which is manifested as an increase in movement distance and time in the open field; Chuang et al. (2017) reported that 4-week treadmill exercise can significantly improve gait speed and balance in the 6-OHDA—induced rat damage model, which is manifested as a significant increase in the contact area of all left paws with the floor, the duration(s) of contact of the left paws with the floor, the swing speed of the left paws, and the distance between successive placements of the same paw, and a significant reduction in the distance between the two hind limbs and the average number of methamphetamine-induced rotations. According to the findings of this study, 4-week moderate-intensity treadmill exercise intervention can significantly alleviate motor dysfunction in the rat PD model, which is manifested as a significant increase in the movement distance, the movement time, and the movement velocity in the open field, and a significant decrease in the number of APO-induced rotations. A great number of clinical and basic studies have verified that many PD therapeutic regimens (drugs or surgeries) can alleviate motor dysfunction due to PD or progression of PD, but not all can improve the striatal DA concentration or the regeneration of dopaminergic neurons in the SNc compact part. Studies indicate that intracerebral injection of glial cell line-derived neurotrophic factor can relieve motor dysfunction due to PD but not increase the striatal DA level (Gash et al., 1996; Tseng et al., 1997). Likewise, deep brain stimulation also cannot increase the striatal DA level of PD patients (Sakellaridis, 2005; Chiken and Nambu, 2014). This study also found that exercise intervention has no significant effect on the loss of dopaminergic neurons and striatal dopaminergic nerve fibers in the SNc compact part of the rat PD model and cannot reverse the loss of striatal DA in the 6-OHDA-induced rat damage model. A lot of studies have indicated that exercise can enhance the DA use efficiency (Kim et al., 2014) or regulate presynaptic DA transporter and postsynaptic DA receptor (Rui et al., 2013), suggesting that exercise intervention's efficacy in alleviating PD symptoms may involve other regulatory pathways or neurotransmitters more than the dopaminergic system. This study indicates that exercise intervention's efficacy in relieving motor dysfunction due to PD may be correlated with the changes in the release of striatal Glu and acetyl choline (Sun et al., 2012). Therefore, exercise's efficacy in alleviating motor dysfunction in the rat PD model is correlated with its effect on glutamatergic transmission and other systems rather than its direct effect on the loss of DA.

A study suggested that the loss of striatal dopaminergic neurons in the SNc causes the increase of striatal glutamatergic transmitters (Mao et al., 2013; Litim et al., 2017;

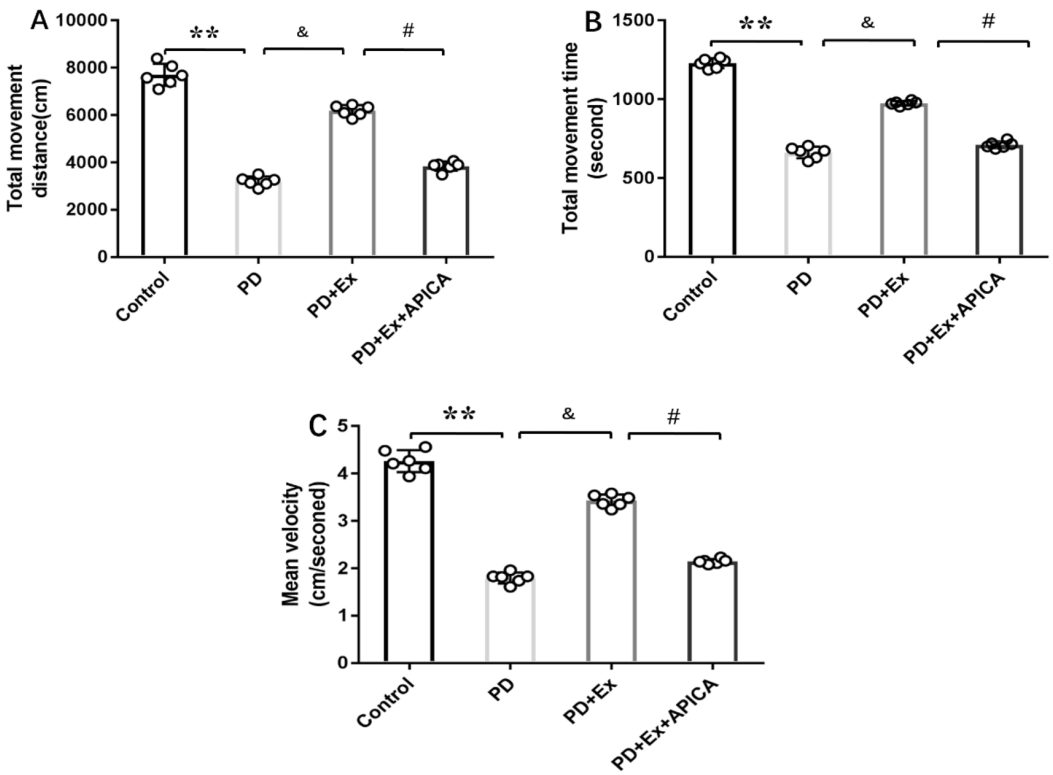

FIGURE 10 | Effect of mGluR2/3 antagonist (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA) on locomotor activity of exercise-mediated rat PD model. **(A)** Effect of mGluR2/3 antagonist APICA on movement distance of exercise-mediated rat PD model. **(B)** Effect of mGluR2/3 antagonist APICA on movement time of exercise-mediated rat PD model. **(C)** Effect of mGluR2/3 antagonist APICA on mean velocity of exercise-mediated rat PD model. Compared with the control group, $^{**}P < 0.01$; compared with the PD group, $^{\&}P < 0.05$; compared with the PD + exercise group, $^{\#}P < 0.05$.

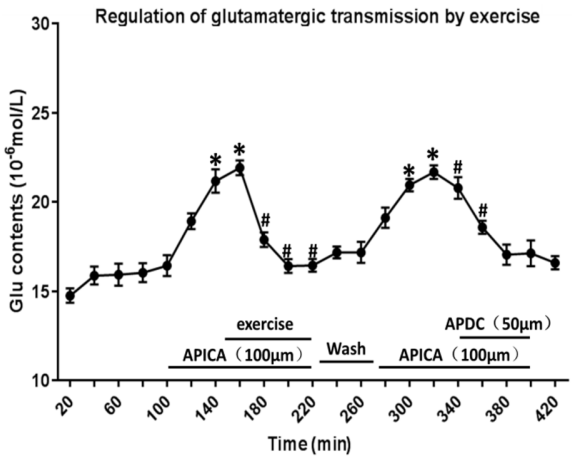

FIGURE 11 | Effect of exercise on mGluR2/3 antagonist-induced extracellular Glu concentration in the striatum. mGluR2/3 antagonist APICA (100 μm) can increase extracellular Glu content in the striatum of normal rats. Exercise intervention or mGluR2/3 agonist aminopyrrolidine-2, 4-decarboxylate (APDC; 100 μm) can significantly reduce the increased concentration in the striatal extracellular Glu induced by mGluR2/3 antagonist ($n = 6$). Compared with the baseline, $^{*}P < 0.05$; compared with APICA, $^{\#}P < 0.05$.

Bagga et al., 2018). Specifically, the increase of cortex–striatum glutamatergic pathway activity and the higher extracellular Glu level in striatal neurons are correlated with the depletion of DA (Ossowska et al., 2002; Ambrosi et al., 2014; Melief et al., 2018; Jamwal and Kumar, 2019). On the basis of the findings of this study, the damage of dopaminergic neurons leads to the increase of extracellular Glu level in striatal neurons, which conforms to the findings of previous studies. This verifies that the damage of dopaminergic neurons can strengthen striatal glutamatergic transmission and involve cortex–striatum glutamatergic projection neurons in pathophysiology of PD. Therefore, the reversion of abnormal cortex–striatum glutamatergic transmission is regarded as one effective means to treat or alleviate PD. According to the findings of this study, exercise intervention can significantly reduce the extracellular Glu level in striatal neurons and improve the locomotor activity in rats. Furthermore, the locomotor activity in rats is negatively correlated with the extracellular Glu level in striatal neurons, which is more significant at the fourth week and the second week. This indicates that the inhibition of cortex–striatum glutamatergic transmission mediates the efficacy of exercise intervention in alleviating motor function in the rat PD model to some extent.

Effect of GluR2/3 in Alleviating Motor Dysfunction in Rat PD Model

As the primary excitatory neurotransmitter in the central nervous system, Glu mainly exerts biological effects by combining with the corresponding receptors in the synaptic membrane. Glu receptors are classified into iGluRs and mGluRs (Lau and Tymianski, 2010; Lewerenz and Maher, 2015). In recent years, extensive studies have focused on the effect of iGluRs in the occurrence and development of PD and put forward that the excitotoxicity of Glu may be one of the important mechanisms in the occurrence and development of PD (Chotibut et al., 2014). Many studies have indicated that although iGluR antagonist has an anti-PD effect, it is still restricted because the receptor is not specifically distributed in the central nervous system, and nonselective iGluR antagonist may have significant side effects, like cognitive dysfunction and psychotomimetic symptoms, in clinical experiments (Du and Chen, 2017; Masilamoni and Smith, 2018). Therefore, researchers have turned to mGluRs and found that mGluR2/3 may be an important target for the treatment of PD (Chan et al., 2010). A study has suggested that mGluR2/3 is located on the presynaptic membrane as an autoreceptor for negative feedback control of Glu transmission, and its activation can reduce Glu release (Marino et al., 2002; Gasparini et al., 2013; Amalric, 2015). Currently, mGluR2/3 agonist has been partially applied in clinical treatment, with a significant efficacy (Samadi et al., 2009). A study showed that selective mGluR2/3 agonist reduced the overreaction of cortex–striatum nerve fibers after dopaminergic denervation (Conn et al., 2005; Litim et al., 2017). Senkowska and Ossowska (2003) reported that the activation of mGluRs in group II could relieve musculoskeletal rigidity and bradykinesia in the rodent PD model. Murray et al. (2014) found that injection of mGluR2/3 agonist in the lateral ventricles or SNc relieves reserpine-treated rats or 6-OHDA-induced rat damage models. Chang et al. (2006) reported that injection of mGluR2/3 agonist in the SNc could significantly reduce the forelimb application dissymmetry percentage and the net number of rotations toward the opposite side in the 6-OHDA rat model. According to the findings of this study, exercise intervention significantly increased the striatum mGluR3 mRNA expression level on the 6-OHDA-induced damage side and significantly upregulated the striatum mGluR2/3 protein expression on the damage side. Therefore, exercise intervention can strengthen the mGluR2/3 activity at local sites, and this is mainly achieved by affecting the striatal mGluR3 mRNA. It is inferred that the increased mGluR2/3 activity may automatically inhibit the presynaptic Glu release, so as to cause a lower extracellular Glu concentration and the changes in motor dysfunction. On the basis of the findings of this study, exercise intervention significantly reduced the concentration of extracellular Glu in striatal neurons in the 6-OHDA-induced rat damage model and significantly improved the locomotor activity of model rats. This study focused on exercise intervention in the rat PD model, as well as the administration of mGluR2/3 antagonist to the striatum at the 6-OHDA-induced damage side through the microinjection pump. Based on the findings, injection of mGluR2/3 antagonist in the striatum increased the concentration of extracellular Glu in striatal neurons and prevented the efficacy of exercise in alleviating motor dysfunction in the rat PD model. Therefore, mGluR2/3 participated in the exercise-mediated motor dysfunction alleviation in the rat PD model. This study provides direct evidence for the effect of mGluR2/3 in preventing the Glu release and the efficacy of exercise in preventing the cortex–striatum Glu release capability at the presynaptic terminal by increasing the local mGluR2/3 expression in the striatum.

CONCLUSION

Exercise intervention can significantly alleviate motor dysfunction in the rat PD model, upregulate the striatal mGluR2/3 protein expression, and reduce the Glu concentration. mGluR2/3 antagonist can significantly increase extracellular Glu in striatal neurons and offset the beneficial effect of exercise in alleviating motor dysfunction in the rat PD model. Exercise intervention may exert an effect in alleviating motor dysfunction in the rat PD model by upregulating the striatal mGluR2/3 protein expression, reducing the Glu release at the presynaptic terminal, and relieving the excitotoxicity to the postsynaptic membrane.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental animal ethics committee, school of physical education and sports, Beijing normal university.

AUTHOR CONTRIBUTIONS

PC: complete specific experiments and thesis writing. XL: the data processing.

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STUDY PROTOCOL
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The Effect of High-Intensity Interval/Circuit Training on Cognitive Functioning and Quality of Life During Recovery From Substance Abuse Disorder. A Study Protocol

Øyvind Andreassen[1,2], Kolbjørn Brønnick[3,4], Anne-Lill Njå[2], Einar Furulund[2] and Sverre Nesvåg[2,3]*

[1] Salvation Army Treatment Center Stavanger, Stavanger, Norway, [2] Center for Alcohol and Drug Research, Stavanger University Hospital, Stavanger, Norway, [3] Department of Public Health, Faculty of Health Sciences, Stavanger, Norway, [4] Centre for Age-Related Medicine (SESAM), Stavanger University Hospital, Stavanger, Norway

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***Correspondence:**
Øyvind Andreassen
oyandreassen@gmail.com

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This proposed study will examine whether structured physical activity reduces the recovery time of cognitive functioning during the early phase of substance use disorder treatment. Addiction or substance dependence is associated with neurobiological changes and cognitive impairment that can affect quality of life and the efficacy of therapy for up to a year after clinical detoxification. The biological, psychological, and social effects of physical exercise have the potential to be a therapeutic approach to increase quality of life and relieve symptoms associated with substance abuse, such as psychosis, depression, and anxiety. There is a dearth of research on physical activity and exercise in clinical substance use disorder patients. This protocol describes a clinical study that will examine cognitive recovery after substance abuse using physical exercise as a treatment intervention. We will use a quasi-experimental longitudinal clinical trial, with a pretest and multiple posttests, on naturally randomized sequential groups. Patients will be consecutively be recruited into the study groups, with a control group that is completed, before its followed by an intervention group, each with 30 patients. Patients will be enrolled 2 weeks after the start of detoxification, at which time all subjects will be inpatients at the Stavanger Salvation Army Treatment Center in the Norwegian specialized healthcare system. Cognition will be evaluated with a comprehensive battery of cognitive tests, including several tests of executive function. Physical fitness will be tested with the Rockport 1-Mile Walk Test, the 30-S Chair Stand Test, the 1-Min Burpee Test at baseline (within the first 2 weeks of admittance) and after 4 weeks. The intervention will be a 30-min workout at 70–90% of maximum heart rate (134–170 bpm), recorded and calculated by a Polar heart rate monitor. The intervention treatment will be administered four times a week for 4 weeks and will consist of high-intensity circuit training, high-intensity interval training, functional movement, and primitive reflex training. We anticipate improvement in both the control and intervention

groups, with the exercise intervention group having the greatest increase in recovery of cognitive function because of the combination of functional full body movements and primitive movement training in an intense interval training program.

Clinical Trial Registration ID: ISRCTN74750479, Retrospectively Registered.

Keywords: physical activity, high-intensity interval training, high-intensity circuit training, substance use disorder, brain health, neurocognition

INTRODUCTION

Substance use disorders (SUD) are among the most prevalent, chronic, and severe diseases in the world in terms of mortality and disability (Kessler et al., 2007), and they are associated with considerable social, economic, and individual health consequences (Adrian and Barry, 2003; Schuckit, 2006; Hasin et al., 2007; Kirby and Sugden, 2007; Wittchen et al., 2011). The combination of impaired physical and mental health among SUD populations is associated with life expectancies that are 20–30 years less than the general population (Stenbacka et al., 2010; Nordentoft et al., 2013). Individuals with SUD have a high tendency for comorbidity. Compared with the general population, the SUD group has more frequent contact with the healthcare system (Richards et al., 1999, 2017; Bernstein et al., 2014) because of a variety of illnesses such as cancer, diabetes, cardiovascular diseases, different kinds of physical trauma, and suicide (Kim et al., 2009; Stenbacka et al., 2010; Nordentoft et al., 2013). Depression, anxiety, attention deficit hyperactivity disorder (ADHD), personality disorder, and psychosis are among the most common comorbidities (Conway et al., 2006; Pennay et al., 2011; Pettinati et al., 2013; Adida et al., 2014; Harstad et al., 2014; Hartz et al., 2014). Individuals with SUD also have a high prevalence of cognitive impairment, which can contribute to low quality of life and the tendency to drop out of treatment (Rosselli and Ardila, 1996; Gouzoulis-Mayfrank and Daumann, 2006; Hagen et al., 2016, 2017a,b, 2019; Topiwala et al., 2017; Hall et al., 2018). A recent study (Hagen et al., 2016) also highlighted the importance of adequate recovery time for cognitive functions. Although pharmacological and psychological interventions are well established in the treatment and the research fields, relapse rates are typically high. The limited success rates of SUD treatments provide a good argument for focusing on adjunct treatments for SUD patients with interventions that target both physical and mental health.

Physical activity may be an excellent adjunct treatment to existing treatment regimens by improving the patient's function and reducing symptoms. Exercise is a subcategory of physical activity that is planned, structured, repetitive, and designed to improve or maintain physical fitness, physical performance, or health (Caspersen et al., 1985). The general effects of physical activity are most significant when participants transition from an inactive lifestyle to a more physically active lifestyle (Pate et al., 1995; Ekelund et al., 2016). Exercise-based interventions are well established in the mental health service and research fields. Increased physical fitness, positive effects on depression and anxiety symptoms, and fewer negative symptoms for patients with schizophrenia are some of the results from mental health research (Rosenbaum et al., 2014; Haglund et al., 2015). Exercise interventions have also been observed to have positive effects on cognitive functions for severe mental illnesses (Firth et al., 2016), perhaps partially through an impact on brain functioning. Such research constitutes a persuasive argument for the exploration of exercise-based interventions in chronic or severe SUD populations.

However, only a few studies have examined the impact of physical activity or exercise in patients with SUD (Zschucke et al., 2012; Giesen et al., 2015). In September 2017, we conducted a literature search in Embase, Medline, SPORTDiscus, PsycINFO, and CINAHL using the search terms "high-intensity interval training" (HIIT), "high-intensity circuit training" (HICT), "cognitive function," and "SUD." The results of our search yielded 11 articles (Weinstock et al., 2008, 2014; Smith and Lynch, 2011; Wolff et al., 2011; Zschucke et al., 2012, 2013; Flemmen et al., 2014; Wang et al., 2014; Haglund et al., 2015; Klika and Jordan, 2015; Unhjem et al., 2016) of relevance to this study. This corpus included research that examined physical activity for SUD populations as outpatients or inpatients in recovery after withdrawal (Zschucke et al., 2012). Physical activity or exercise for SUD populations was shown to improve physical fitness, cardiovascular health, and sleep quality, and reduce the withdrawal symptoms of substance use, as well as anxiety and depression symptoms (Roessler, 2010; Zschucke et al., 2012; Wang et al., 2014; Giesen et al., 2015; Hallgren et al., 2017). It has also been observed that engaging in a physical activity program during treatment can contribute to reduced dropout rates, thereby increasing the success rate of therapy (Weinstock et al., 2014). Although this body of work presents promising results, many of the studies have methodological limitations, such as being pilot studies or lacking control groups. Because of the positive impact exercise can have on mental health, we believe it is extremely important to explore exercise interventions in the clinical treatment and recovery for SUD populations. Preexisting data and approaches in the field of mental health and SUD show great promise; for example, HIIT principles have been found to increase health benefits (Milanović et al., 2015; Unhjem et al., 2016; Karlsen et al., 2017). However, we lack data on dose, intensity, and the most beneficial types of exercise.

1. This study protocol is designed to investigate the impacts of HIIT and HICT combined with functional exercises and primitive reflex training on quality of life and cognitive functioning for patients with SUD. Specifically, the protocol will examine whether structured physical

activity improves cognitive functioning for SUD patients relative to that of SUD patients in a control group.
2. We will examine whether different intensities of physical activity, such as HIIT/HICT versus other types of physical activity (e.g., hiking) are associated with different cognitive recovery trajectories during the subacute phase of SUD treatment.

This research protocol has several possible applications. First, it will enable future studies to reproduce the exercise protocol and increase the knowledge base on SUD and the effects of exercise. One of the more significant problems in the exercise literature is the inadequate description of exercise, such as the dose, intensity, and how exercises are implemented in studies, which limits reproducibility. This protocol and training regime would serve as a base for comparisons with other workout routines, types of exercises, and duration of exercises. Because this exercise regime will be compared with control group activities, it will be validated as having positive or negative effects for SUD patients in a subacute clinical setting.

Second, because there are so few studies on exercise and substance abuse, a protocol will make it easier to reproduce and increase the number of studies with identical exercise regimes by using this protocol as a guide. This would facilitate comparisons between studies and allow for systematic reviews and meta-analyses.

Third, if this protocol leads to an effective intervention which improves the participants' cognitive functioning and quality of life, the training regime would be inexpensive and easy to implement in a clinical setting, as there is no need for specialized equipment or extensive staff training.

MATERIALS AND EQUIPMENT

Design

The design of this study is a quasi-experimental, longitudinal clinical trial, with pretest and multiple posttests. The control and intervention groups will be run sequentially, with patients naturally randomly assigned to substance abuse treatment (see below). This study will be conducted in a clinical setting as part of the daily treatment interventions conducted at the Salvation Army Treatment Center, Stavanger (FAB), including testing the efficacy of HIIT for the recovery of cognitive functioning in patients with SUD. After completion of the clinical trial, patients will be followed prospectively for 1 year.

Control Group

This study will have an active control group of SUD inpatients recruited from the same treatment facility as the patients in the intervention group. The control group will receive treatment as usual (TAU) for SUD patients. The treatment focuses on activities of daily living (ADL), including personal hygiene (e.g., washing oneself and brushing teeth); sleep hygiene, which focuses on regular sleep and wake-up times to help normalize sleep patterns; learning or relearning how to prepare and cook food; eating in a social setting at fixed times; house cleaning and work assignments relevant to making the ward run smoothly; structured socializing and group sessions, twice a day; and structured physical activity in the form of 30–120-min daily hikes and a longer weekly hike for a duration of 3–5 h.

Intervention Group

Participants in the intervention group will receive the same TAU as the control group, and they will also do four additional 30-min exercise protocols each week.

Randomization

Although we will not be conducting a true randomized control trial, the control and intervention groups will be run consecutively, and random assignment to these groups will occur naturally because the external allocation system of three separate units allocates patients with different needs to the SUD treatment waiting list independently of each other. Natural allocation will be aided further by SUD patients dropping out of the waiting list for entry to treatment.

Ethics Statement

This research project was approved by the Regional Ethics Committee for Medical Research Ethics, Western Norway (2011/1877). Written consent is collected from all participants.

All sensitive data (digital and non-digital) generated are confidential and will be treated according to the standards set by the Norwegian Data Inspectorate (Datatilsynet) and in compliance with the Health Research Act and the Personal Data Act.

Equipment

Location: Salvation Army Treatment Center Stavanger, Rogaland, Norway.

Watch (Polar M200, M360), weight scale, stopwatch, chair, running track (Stavanger Stadium), exercise sling, two gym mats, computer for neurocognitive testing, and a timer (Tabata).

STEPWISE PROCEDURES

Recruitment and eligibility. Patients will be recruited from the Helse Vest catchment area via the subacute treatment facility FAB. The Helse Vest catchment area consist of western Norway. FAB is mainly recruiting form the county of Rogaland and the main cities in the region Stavanger, Sandnes, and Haugesund. The patient target group for FAB is refered patients from the specialized health care system, with an addiction so serious that inpatient treatment is deemed necessary by a evaluation center. Comorbidity of light psychiatric diagnoses are accepted. We included patients of 18 years of age and upward. The patients often receive social benefits or disability pension. Education among the patient group is mixed from elementary school to university educated. We included patients mixed between first time rehabilitation to varying number of previous admission. Eligibility: SUD diagnosis, admitted as an inpatient to FAB; $\geq$18 years of age; passes the physical evaluation for the institution to perform intensive training; no medical history or illness

that precludes participation in physical activity; and psychiatric diagnoses no more serious than the grading of light to moderate. Patients will be excluded from the study if their medical history (e.g., physical disability, disease, or injury) could interfere with or be exacerbated by physical activity. For example, patients with paralysis, severe pain, obstructive disease, glaucoma, or who are unable to sit, stand, and/or walk will be excluded, as well as patients with a severe cognitive deficit, such as dementia. A severe cognitive deficiency will be assessed based on the patients' responses to two questions: "Do you have trouble with your memory that affects your daily life?" and "Have you been given a medical diagnosed of dementia?" Protocol deviations: If participants are absent from more than 60% of the training sessions. They are considered protocol violators. If they are not able to participate at allocated test times and complete the tests, they are considered protocol violators. They are still included in the study and tested as soon as possible. Statistical analyses will be performed both based on the principle of "intention to treat" including everyone regardless of protocol violation and "per protocol" analyses will be performed, comparing only those that have completed the protocol.

1. Baseline testing. The physical and neuropsychological testing will be conducted within 2 weeks of admittance to the Salvation Army Treatment Center.
2. The researchers will not conduct randomization, as there is natural allocation as a result of the SUD treatment allocation system, which will be continuous throughout the study.
3. The control group is completed first. Structure is changed in the clinic and intervention group is started up after the last control patient has left the clinic.
4. The intervention will consist of the physical exercise protocol, which will be conducted for 4 weeks.
5. The final tests of physical function will be conducted the week after the exercise protocol is completed. The prospective follow-up will consist of cognitive testing carried out at 3, 6, 9, and 12 months after the baseline test.
6. Discontinuation: Patients that don't follow the protocol or don't follow the TAU of the Facility will be discontinued. If they don't complete the follow up test with in the allocated time line or neglect to perform the test they will be discontinued (**Figure 1**).

Testing Procedures

Rockport 1-Mile Walk Test

This will be performed at the Stavanger outdoor stadium. Patients will be timed while walking 4 × 400 m, and pulse rate will be recorded with the final crossing of the finish line (George et al., 1998).

30-Second Chair Stand Test

This will be conducted by having the patient perform as many sit–stand–sit cycles as possible during a 30-s test (Macfarlane et al., 2006; Millor et al., 2013), with the number of cycles completed recorded. This test will be conducted at the Salvation Army Treatment Center.

1-Minute Burpee Test

This is a functional test and outcome measure. The burpee is a total body exercise used in strength training, high-intensity training, and aerobic exercise. It is designed to develop strength, agility, coordination, and aerobic performance. The test starts with the subjects in a standing position with their feet shoulder-width apart. They then drop into a squat position with their feet underneath them and their hands on the ground and quickly extend their feet in one motion to assume the front plank position with legs completely extended and back straight. Finally, they return to the squat position and then jump straight into the air as high as possible. Repeat. This entire exercise is intended to be performed in a fluid, rapid movement (Gist et al., 2014).

Physical Exercise/Intervention Protocol

The study uses HIIT/HICT consisting of functional exercise movements combined with primitive reflex training exercises conducted in a 4-week program to examine the effects of this protocol on the recovery of cognitive function after chronic SUD. In the current literature, high-intensity training is considered a high-yield training form and usually includes three types of exercise (Unhjem et al., 2016). The best evidence indicates that a minimum of three 30–60-min sessions are needed per week (Milanović et al., 2015; Eddolls et al., 2017), over the course of 4–9 weeks of training. Because our patient population has relatively short-term stays in the facility, we set the control and intervention periods as 4 weeks each. As noted, the physical exercise protocol for the intervention group consists of four 30-min sessions per week, with an active warm-up included in each session. Each of the 30-min exercise sessions will be the same, with a target heart rate of 70–90% of the patient's maximum heart rate (220 beats per minute minus the patient's age). Each training session consists of a warm-up period at 60–70% of maximum heart rate (114–133 bpm), followed by exercise performed at 70–90% of maximum heart rate (134–170 bpm), with a cutoff at 90% (171 bpm), and then an active cooldown period. Polar heart rate zones will be used to control the exercise intensity throughout the training sessions. Because of the low cost and limited need for equipment, we plan to use body weight functional exercises in this study. The exercises for each training session will be performed at nine stations, with 45 s of work at each station and 15 s to rest and change stations. Participants will complete three circuits of the nine stations, with 25 s of rest between circuits. The active cooldown consists of walking for 5 min. The nine exercises are: air squats; cat/camel; reverse rowing with a sling; starfish; burpees; frog sit-ups/CrossFit sit-ups; crawling [asymmetrical tonic neck reflex (ATNR) exercise]; jumping jacks; and push-ups. **Supplementary Appendix A** contains further description and information about the exercises in the protocol.

Neuropsychological Testing

The present study is an intervention study as a follow up study from the Stayer longitudinal cohort study, and we will be using the some of the cognitive tests, that the Stayer study uses to follow the mental health of their participants. The test is listed below. All the neuropsychological testing will be performed by a research assistant who is specially trained to administer

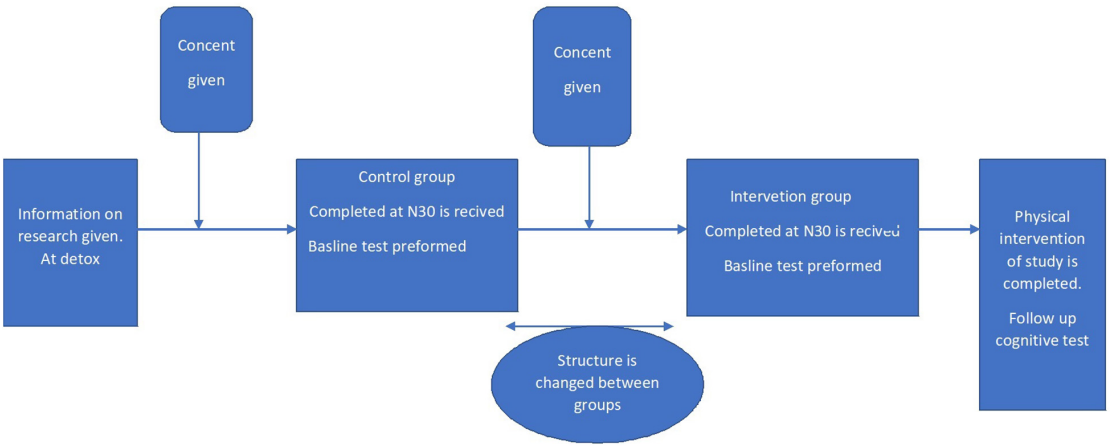

FIGURE 1 | FAB, Stavanger 2019.

neuropsychological tests. The following paragraphs summarize the cognitive tests.

Clinical Self-Rating Questionnaires

The Symptom Checklist-90-Revised (SCL-90-R) is a 90-item self-report symptom inventory developed by Derogatis (Derogatis and Unger, 2010) in the mid-1970s to measure psychological symptoms and psychological distress. It is designed for use with individuals from the community, as well as with individuals with either medical or psychiatric conditions. The SCL-90-R assesses psychological distress along nine primary symptom dimensions and three summary scores, which are referred to as global scores. The principal symptom dimensions are labeled Somatization (SOM), Obsessive-Compulsive (OBS), Interpersonal Sensitivity (INT), Depression (DEP), Anxiety (ANX), Hostility (HOS), Phobic Anxiety (PHOB), Paranoid Ideation (PAR), and Psychoticism (PSY). The three global measures are the Global Severity Index (GSI), the Positive Symptom Distress Index (PSDI), and the Positive Symptom Total (PST) (Derogatis and Unger, 2010).

The Satisfaction with Life Scale (SWLS) assesses subjective well-being by focusing on global life satisfaction. It does not tap related constructs such as positive affect or loneliness. The SWLS has favorable psychometric properties, including high internal consistency and high temporal reliability. Scores on the SWLS correlate moderately to highly with other measures of subjective well-being and correlate predictably with specific personality characteristics (Diener et al., 1985; Pavot et al., 1991).

The Behavior Rating Inventory of Executive Function–Adult Version (BRIEF-A) is a self-report form designed to be completed by adults 18–90 years of age, including adults with a wide variety of developmental, systemic, neurological, and psychiatric disorders, such as attention disorders, learning disabilities, autism spectrum disorders, traumatic brain injury, multiple sclerosis, depression, mild cognitive impairment, dementia, and schizophrenia. The BRIEF-A is composed of 75 items in nine non-overlapping and theoretically and empirically derived clinical scales that measure various aspects of executive functioning (Gioia et al., 2000; Kessler et al., 2005).

The Adult ADHD Self-Report Scale (ASRS) include 18 questions about the frequency of recent the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) Criterion A symptoms of adult ADHD. The ASRS screener includes six out of these 18 questions that were selected based on stepwise logistic regression to optimize concordance with the clinical classification. ASRS responses were compared to blind clinical ratings of DSM-IV adult ADHD in a sample of 154 respondents who previously participated in the US National Comorbidity Survey Replication (NCS-R), oversampling those who reported childhood ADHD and adult persistence (Reitan and Wolfson, 2004).

The Alcohol Use Disorders Identification Test (AUDIT) consists of a 10-item Core questionnaire and an 8-item Clinical procedure. AUDIT was designed to identify hazardous drinkers (whose drinking increases their risk of alcohol-related problems, although alcohol-associated harm has not yet occurred); harmful drinkers (who have had recent physical or mental problems related to their drinking, but who are not alcohol-dependent); and people with alcohol dependence (Tombaugh, 2004).

The Drug Use Disorders Identification Test (DUDIT) is an 11-item self-report questionnaire developed to screen individuals for drug problems (Gioia et al., 2000).

The quality register is a national semi-structured interview for the Norwegian healthcare system that is used as a tool for patient feedback and the mapping of patient health, quality of life, employment, housing, and education.

Neuropsychological Tests

The Montreal Cognitive Assessment (MoCA) (Voluse et al., 2012) takes approximately 10 min to administer and is designed to detect mild cognitive impairment in elderly participants who score in the normal range on the Mini-Mental Status Examination. The MoCA uses 30 items to assess multiple cognitive domains, including: short-term memory (5 points); visuospatial abilities via clock drawing (3 points), and a

cube copying task (1 point); executive functioning via an adaptation of the Trail Making Test Part B (1 point), phonemic fluency (1 point), and verbal abstraction (2 points); attention, concentration, and working memory via target detection (1 point), serial subtraction (3 points), digits forward (1 point), and digits backward (1 point); language via confrontation naming with low-familiarity animals (3 points) and repetition of complex sentences (2 points); and orientation to time and place (6 points) (Voluse et al., 2012). The MoCA is scored by obtaining an item total, and the authors recommend a clinical cutoff score of 26 (Voluse et al., 2012).

The Stroop Color and Word Test (SCWT) is used extensively to assess the ability to control the cognitive interference that occurs when the processing of a specific stimulus feature impedes the simultaneous processing of a second stimulus attribute, which is well known as the Stroop effect. The Stroop test has been used in previous trials on physical exercise and cognition and is a sensitive measure of prefrontally mediated cognitive functions (Bohn et al., 1995; Nasreddine et al., 2005).

The Trail Making Test is a test of visual attention and task switching. It consists of two parts in which the subject must connect a set of 25 dots as quickly and accurately as possible (Golden and Freshwater, 1978). The test provides information about visual search speed, scanning, speed of processing, and mental flexibility, as well as executive functioning (Arnett and Labovitz, 1995; Arbuthnott and Frank, 2000; Martínez et al., 2016; Scarpina and Tagini, 2017).

ANTICIPATED RESULTS

In this study, we expect the participants in both the control and intervention groups to show progress in physical and mental health after a period of substance abuse. We believe that the data generated in this project will mostly be at the interval or ratio level (whether the rating of perceived exertion is ordinal or interval data will not be discussed here). We will use the mean as a measure of central tendency for the group results for the neurocognitive test, while the reflexes we use frequency and proportions, and the standard deviation to express the spread of results within the group. If useful, the standard error of the mean (SEM) will be used to express the accuracy of the mean value. If the results are highly skewed (skewness > 1.5), we will use a log transformation and geometric mean together with a 95% confidence interval to express central and spread tendencies. To compare results over time from pretest to posttests, and between-group differences, we will use repeated measures ANOVA/*t*-tests with Sidak/Holm *post hoc* tests, where we have collected three or more measurements a linear mixed modeling accounting for the missing data will be used. Group comparisons will be made with an ANOVA/*t*-test for independent groups. If the test of normality should fail (Kolmogorov–Smirnov with Lilliefors correction), the corresponding non-parametric tests will be used, i.e., the Wilcoxon–Mann–Whitney rank sum test for independent *t*-tests and repeated measures ANOVA for ranks (Tukey's *post hoc* test). Statistical significance will be set at a *p*-value of ≤ 0.05, and effect sizes will be calculated according to Cohen (1988), with the help of an online effect size calculator (the CEM effect size calculator, Center for Evaluation and Monitoring, Durham University, United Kingdom[1]). An effect size of ≥ 0.5 will be considered clinically relevant (two-sided). If there is a need for building indexes of different types of scores, *Z*-scores will be used. Based on previously published data on fitness development and depression scores, we should be able to attain statistical power of 0.8 with at least 10 subjects in each of the two groups. Nevertheless, we have set target recruitment at $n = 30$ for each group.

DISCUSSION

This study is the first of its kind where investigations of changes in cognitive function as a direct effect of structured physical activity are assessed when the physical exercises are based on bodyweight exercises and functional movement structed into a high-intensity circuit. We hypothesize that the benefits of doing it this way will kick start the body's own regeneration process and brain plasticity to repair the damage that has occurred do to drug use due to release of signaling substances such as brain-derived neurotropic factor. In conducting this exercise program, we observe changes in both groups.

Limitations of the Study

Because the intervention and control groups will be run consecutively rather than concurrently, there is the potential for non-equivalent time periods to create bias. However, we chose this approach because previous studies had suffered recruitment failures and high dropout rates. The length of the proposed study is not fixed, and because of our funding and support, this study can continue until the quota for participants is met.

This study is designed to accommodate clinical realities. Thus, different SUD groups are not fractionated during treatment, and the project protocol does not divide different groups of SUD after clinical classification. This may create some problems with the results from baseline because of various issues with the abuse of different drugs and possible side effects that might affect the test results differently. We will account for this Statistically.

Finally, the patient population will be recruited straight out of a detoxification clinic, which will most likely influence the test results. However, this may actually be an advantage, because having access to patients so quickly after controlled, medically supervised detoxification may give us a more accurate picture of their mental status when the patients are at their lowest point in their recovery trajectories.

The Norwegian Ministry of Health and Social Affairs and the Salvation Army of Norway have provided financial support for this study.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regionale komiteer for medisinsk

[1]http://www.cemcentre.org/evidence-based-education/effect-size-calculator

og helsefaglig forskningsetikk (2011/1877/REK vest). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors conceived the study. ØA wrote the manuscript and contributed with scientific/clinical expertise. EF and KB also contributed to writing this manuscript. SN contributed to project oversight and resource control. A-LN contributed to designing the protocol and retrieval of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2019.02564/full#supplementary-material

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Aerobic Exercise Training Improves Cerebral Blood Flow and Executive Function: A Randomized, Controlled Cross-Over Trial in Sedentary Older Men

*Jordi P. D. Kleinloog[1], Ronald P. Mensink[1], Dimo Ivanov[2], Jos J. Adam[1], Kamil Uludağ[3,4] and Peter J. Joris[1]**

[1] Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, Netherlands, [2] Department of Cognitive Neuroscience, Faculty of Psychology and Neuroscience, Maastricht University, Maastricht, Netherlands, [3] Department of Biomedical Engineering, N Center, Sungkyunkwan University, Suwon, South Korea, [4] Techna Institute & Koerner Scientist in MR Imaging, University Health Network, Toronto, ON, Canada

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Henriette van Praag, Florida Atlantic University, United States

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***Correspondence:**
Peter J. Joris
p.joris@maastrichtuniversity.nl

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Background: Physical activity may attenuate age-related cognitive decline by improving cerebrovascular function. The aim of this study was therefore to investigate effects of aerobic exercise training on cerebral blood flow (CBF), which is a sensitive physiological marker of cerebrovascular function, in sedentary older men.

Methods: Seventeen apparently healthy men, aged 60–70 years and with a BMI between 25 and 35 kg/m^2, were included in a randomized, controlled cross-over trial. Study participants were randomly allocated to a fully-supervised, progressive, aerobic exercise training or no-exercise control period for 8 weeks, separated by a 12-week wash-out period. Measurements at the end of each period included aerobic fitness evaluated using peak oxygen consumption during incremental exercise (VO_{2peak}), CBF measured with pseudo-continuous arterial spin labeling magnetic resonance imaging, and post-load glucose responses determined using an oral glucose tolerance test (OGTT). Furthermore, cognitive performance was assessed in the domains of executive function, memory, and psychomotor speed.

Results: VO_{2peak} significantly increased following aerobic exercise training compared to no-exercise control by 262 ± 236 mL ($P < 0.001$). CBF was increased by 27% bilaterally in the frontal lobe, particularly the subcallosal and anterior cingulate gyrus (cluster volume: 1008 mm^3; $P < 0.05$), while CBF was reduced by 19% in the right medial temporal lobe, mainly temporal fusiform gyrus (cluster volume: 408 mm^3; $P < 0.05$). Mean post-load glucose concentrations determined using an OGTT decreased by 0.33 ± 0.63 mmol/L ($P = 0.049$). Furthermore, executive function improved as the latency of response was reduced by 5% ($P = 0.034$), but no changes were observed in memory or psychomotor speed.

Conclusion: Aerobic exercise training improves regional CBF in sedentary older men. These changes in CBF may underlie exercise-induced beneficial effects on executive function, which could be partly mediated by improvements in glucose metabolism. This clinical trial is registered on ClinicalTrials.gov as NCT03272061.

Keywords: aging, arterial spin labeling, cerebral blood flow, cognition, exercise, glucose metabolism

INTRODUCTION

People over the age of 60 years represent 13% of the global population and this number is expected to increase at a rate of approximately 3% per year (Department of Economic and Social Affairs Population Division, 2017). Aging is associated with decreased cognitive performance, which is related to decreased cerebrovascular function (Koekkoek et al., 2015; Leeuwis et al., 2018). As impaired cerebrovascular function may precede the decrease in cognitive performance (Jansen et al., 2016; Wolters et al., 2017; Kleinloog et al., 2018), improving cerebrovascular function is an important target to delay cognitive impairment (Gorelick et al., 2011; Joris et al., 2018). In this respect, interventions to improve cerebral blood flow (CBF), a physiological marker of cerebrovascular function (Brown et al., 2007; Liu and Brown, 2007), are of major interest.

A healthy lifestyle, consisting of a healthy diet combined with increased physical activity has been proposed to protect against cognitive impairment by improving CBF (Gorelick et al., 2011). In fact, CBF was improved following a healthy lifestyle intervention and associated with higher cognitive performance in overweight or obese participants aged between 45 and 76 years (Espeland et al., 2018). Furthermore, cross-sectional studies have observed that lower aerobic fitness in sedentary older individuals was associated with a reduced CBF and decreased cognitive performance (Tarumi and Zhang, 2017). A recent meta-analysis of randomized controlled trials involving adults over the age of 50 years showed that aerobic exercise training improved cognitive performance (Northey et al., 2017). This improvement may relate to changes in CBF, since some studies suggest that CBF in the anterior cingulate and hippocampal brain regions increased following aerobic exercise training in sedentary older individuals (Burdette et al., 2010; Chapman et al., 2013; Maass et al., 2015). These exercise-induced changes in hippocampal CBF were positively related to changes in cognitive memory tasks (Chapman et al., 2013; Maass et al., 2015). Therefore, we concluded in our recent review that increases in CBF may contribute to the beneficial effects of increased physical activity levels on cognitive performance (Joris et al., 2018).

However, well-controlled trials investigating the effect of physical activity on CBF are scarce. In some studies, aerobic fitness was measured using a proxy measure which did not improve (Burdette et al., 2010) or was not measured at all (Consortium TtB, 2017), or improvements in aerobic fitness did not sustain (Chapman et al., 2013). Additionally, two studies primarily focused on the hippocampal region, potentially missing changes outside this region (Burdette et al., 2010; Maass et al., 2016). The objective of the current randomized, cross-over trial was therefore to investigate effects of a well-controlled 8-week aerobic exercise training period on CBF and cognitive performance. The study was performed in sedentary overweight or slightly obese older men, because this cohort has been shown to have a reduced CBF and cognitive performance at baseline (Willeumier et al., 2011; Birdsill et al., 2013; Tarumi and Zhang, 2017; Espeland et al., 2018).

MATERIALS AND METHODS

Study Participants

Apparently healthy overweight or slightly obese men were recruited via posters in university and hospital buildings or advertisement in local newspapers. Additionally, participants who had participated in previous studies at Maastricht University were approached if they had given written consent to contact them again for future studies. Volunteers were invited for a screening visit if they met the following inclusion criteria: aged between 60 and 70 years old, body mass index (BMI) between 25 and 35 kg/m^2; stable body weight (weight gain or loss <3 kg in the past 3 months); non-smoker; no drug or alcohol abuse; no use of dietary supplements known to interfere with the main study outcomes; no diabetes; no use of medication known to affect blood pressure, lipid or glucose metabolism; no severe medical conditions that might interfere with the study (e.g., active cardiovascular disease); and no participation in another biomedical study within 1 month prior to the screening visit.

During screening, sedentary behavior was assessed by means of the international physical activity questionnaire (IPAQ) long version (Craig et al., 2003); an MRI screening list was completed; office blood pressure was measured; a 12-lead electrocardiogram (ECG) was performed; and a fasting blood sample was drawn. Based on the screening results, participants were checked against the following inclusion criteria: classified as low physically active according to the guidelines for IPAQ data processing (IPAQ Research Committee, 2005); no contra-indications for MRI imaging (e.g., any metallic implants or claustrophobia); systolic (SBP) <160 mmHg and diastolic blood pressure (DBP) <100 mmHg, no ECG abnormalities as assessed by a cardiologist; fasting plasma glucose <7.0 mmol/L, fasting serum total cholesterol <8.0 mmol/L, and fasting serum triacylglycerol <4.5 mmol/L. All participants provided written informed consent before screening. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, approved by the Medical Ethics Committee of Maastricht University Medical Center (METC173025), and registered on September 07, 2017 at ClinicalTrials.gov (NCT03272061).

Study Design

The study had a randomized, controlled cross-over design with an 8-week intervention period and an 8-week control period, separated by a 12-week wash-out period. Participants were allocated to start either in the intervention or control period based on a computer-generated randomization scheme. Participants and investigators were unaware of the allocation prior to inclusion but could not be blinded during the intervention and measurements. However, images and blood samples were blinded prior to analysis. During the intervention period, participants followed a fully-supervised, personalized and progressive aerobic-based exercise program on a cycling ergometer for 50 min three times a week. To personalize the program, maximal exercise capacity (VO_{2max}) and maximal workload (P_{max}) were reassessed every 2 weeks and training loads were adjusted accordingly. The program consisted of 10 min warm-up at 45% P_{max}, 30 min at 70% P_{max} and 10 min cool-down at 45% P_{max}. We did not offer a program during the control and wash-out periods, and participants were requested to maintain habitual physical activity levels during the entire trial. Body weight was measured every 2 weeks in the intervention period and every 4 weeks in the control period. Participants were requested to maintain their habitual diet and use of alcoholic beverages throughout the total trial, which was checked using a food frequency questionnaire.

Measurements were performed at the start of the control and intervention periods (baseline; BL), after 4 weeks (WK4) and during two follow-up days (FU) at the end of each period. The first follow-up visit (FU-1) was performed 43 (range: 19–72) hours after the last training. The second follow-up visit (FU-2) was performed 117 (range: 70–118) hours after the maximal exercise test performed during FU-1. A schematic overview of the study design is shown in **Supplementary Figure S1**. On the days preceding measurements, participants were requested to have a regular meal and to refrain from alcohol. Participants arrived after an overnight fast (no food or drink after 08:00 PM, except for water) at the Scannexus research facilities in Maastricht (FU-1) or the metabolic research unit Maastricht (MRUM) (FU-2). Men were asked to come by public transport or by car to standardize measurements as much as possible. All measurements were performed in temperature-controlled rooms at 22°C.

Maximal Exercise Test

Peak oxygen consumption (VO_{2peak}) was assessed every 2 weeks during the intervention period, and three times during the control period at BL, WK4, and FU-1. Heart rate was monitored simultaneously using a chest strap (Sport-tester Polar H10, Kempele, Finland). Thirty minutes before each maximal exercise test, the participants received a small carbohydrate rich meal including a banana and white bread with strawberry jam to optimize performance. The VO_{2peak} test included an incremental step-wise protocol on a calibrated bicycle ergometer (Lode Excalibur Sport 1000W/1.5V, Groningen, Netherlands), while oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured continuously (Omnical, Maastricht University, Netherlands).

The VO_{2peak} test started with a 5-min warm-up at a load of 70W. Workload was increased by 50W every 2.5 min. When anaerobic threshold was observed, workload was increased by 25W every 2.5 min until exhaustion. The anaerobic threshold was determined when the respiratory exchange ratio (RER) was between 0.95 and 1.00. Participants had to reach an RER of at least 1.0 to fulfill the criteria of maximal exertion. P_{max} was calculated as the workload completed ($P_{completed}$) plus time (t) in the last step divided by 150 and multiplied with the load increment of the final stage (ΔW): $P_{max} = P_{completed} + \frac{t}{150} \times \Delta W$. Pedal frequency had to be at least 80 RPM. The exercise test was ceased when the pedal frequency remained below 60 RPM for 10 s. VO_{2peak} was determined as the maximal oxygen consumption for 5 s.

MRI Acquisition

Scans were performed in the morning of FU-1 on a 3T MAGNETOM Prisma Fit MRI-system using a 64-channel head-neck coil (Siemens Healthcare, Erlangen, Germany). Participants were placed in the scanner with their head-first in the supine position. The eye centers were taken as a reference for the magnet isocenter position, which was at the level of the pons to minimize B_0 offsets in the labeling region. The labeling plane was positioned perpendicular to the carotid and vertebral arteries, based on an acquired angiogram (TR 21 ms, TE 7.3 ms, voxel volume 0.9 mm × 0.9 mm × 5.0 mm, 8 degrees flip angle, 26 sagittal slices, duration: 2 min).

Perfusion-weighted images were acquired after an acclimatization period of at least 20 min. During acquisition, participants were asked to look at the center of a displayed black cross to standardize measurements as much as possible and to reduce involuntary movements. Images were acquired using pseudo-continuous arterial spin labeling (PCASL) with background-suppressed segmented three-dimensional (3D) gradient and spin echo (GRASE) readouts. The sequence parameters were: TR 4000 ms, TE 13.6 ms, GRAPPA 2, labeling duration 1750 ms, post-labeling delay 2000 ms, segmentation factor 6 (the scan time per 3D volume was 24 s), and 10 label-control repetitions. The total acquisition duration, including an equilibrium magnetization scan, was approximately 9 min). Nineteen slices with a voxel resolution of 3.0 mm isotropic were acquired. In order to allow CBF quantification, a M_0 image without magnetization preparation and with a TR of 20 s was acquired as well. **Figure 1A** shows a typical perfusion-weighted image that we generated at the Scannexus research facilities in Maastricht.

One high-resolution anatomical 3D magnetization-prepared rapid acquisition with gradient echo (MPRAGE) scan was performed (TR 2400 ms, TE 2.18 ms, TI 1040 ms, 1.0 mm isotropic resolution, 8 degrees flip angle and 160 sagittal slices, duration: 6 min). The field of view across the various sequences was kept constant for accurate registration and anatomical localization.

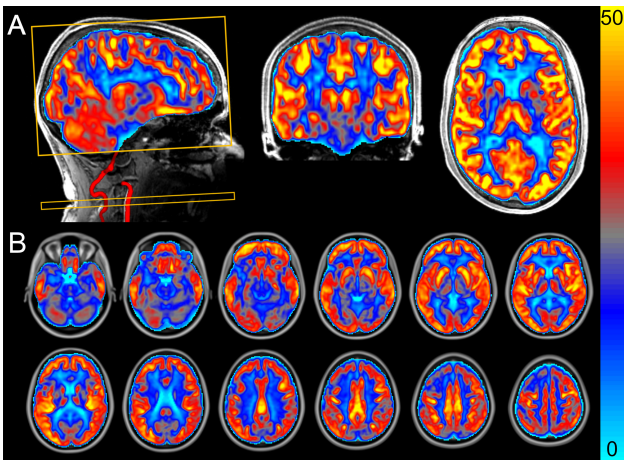

FIGURE 1 | Perfusion-weighted image acquired using pseudo-continuous arterial spin labeling that we generated at the Scannexus research facilities in Maastricht. The images show the cerebral blood flow (CBF) in mL/100 g tissue/min (scale shown by color bar). **(A)** Sagittal slice including angiogram from vertebral and carotid artery, coronal slice, and axial slice. The yellow rectangular boxes represent the imaging box and labeling plane perpendicular to the arteries. **(B)** Mean CBF map from all participants (n = 14) after the control period. Data from a randomized, controlled crossover study with sedentary older men.

MRI Processing

Volbrain was used to perform brain extraction, along with tissue segmentation for the anatomical MPRAGE image (Manjón and Coupé, 2016). Motion correction was automatically performed by Siemens' scanner software. FSL software (Version 6.0) was used to estimate quantitative CBF maps from the ASL data[1]. Pairwise subtraction of label and control images was performed on the PCASL data to generate perfusion-weighted images. Perfusion-weighted images were quantified using the BASIL tool (version 4.0.4) (Chappell et al., 2009). following the recommendations of the ASL White Paper (Alsop et al., 2015) The M_0 image was used for voxel-wise calibration to quantify the perfusion-weighted images. The labeling efficiency was calculated based on the efficiency of four background suppression pulses (0.93^4), which resulted in a cumulative labeling efficiency of 0.64. The T_1 of blood depends on the blood hemoglobin concentrations (ctHb) and was estimated using the following equation: $1000/T_{1a}$ (ms) = 0.016 × ctHb (g/dL) + 0.317 (Li et al., 2017). The used T_1 of gray matter was 1330 ms, while the bolus arrival time was set at 1300 ms.

The calibrated ASL images containing absolute CBF values in mL/100 g tissue/min were co-registered using Boundary-Based Registration to the brain-extracted MPRAGE image using the FLIRT routine (Jenkinson et al., 2002). The gray matter partial volume estimates image was thresholded at 0.6 and binarized to create a mask. The mean gray matter CBF was calculated in the anatomical space by taking the mean CBF over the gray matter mask.

[1]http://fsl.fmrib.ox.ac.uk/fsl

The CBF images in anatomical space were registered to MNI (2 mm) space using a non-linear algorithm (FNIRT) and were used for voxel-wise statistical group comparisons. Voxel-wise analyses was performed to detect significantly changed clusters between the intervention and control periods over the whole brain without prior region of interest (Astrakas and Argyropoulou, 2010).These absolute CBF images in MNI space were spatially smoothed with a Gaussian kernel of 1 mm to account for small regional differences, which still existed across participants. Thereafter, a repeated measures mixed-effects analysis using a general linear model with a single-group paired difference (FLAME stage 1 and 2) was used to generate an image containing Z-scores for each voxel (Woolrich et al., 2004). Cluster information was extracted after correcting for family-wise error using a Z-threshold of 2.3 ($P < 0.05$) and smoothness estimates, which were computed using the Gaussian Random Field model based on the residual error in each participant. The average probability of the location of the significant clusters was determined using the Atlasquery function in combination with the image of the cluster and the Harvard-Oxford (sub)cortical structural atlas.

Blood Sampling and Oral Glucose Tolerance Test

During both periods, fasting blood samples were taken at the same time in the morning from a forearm vein by venipuncture at BL, at WK4, and at FU-1. During FU-2, a 7-point oral glucose tolerance test (OGTT) was performed as a measure of peripheral glucose metabolism. For this, blood samples were taken from an intravenous catheter at baseline (t = 0 min), and 15, 30, 45, 60, 90, and 120 min following ingestion of 75 g glucose (Novolab, Geraardsbergen, Belgium). After blood sampling, NaF-containing vacutainer tubes (Becton, Dickson and Company, Franklin Lanes, NY, United States) were immediately placed on ice and centrifuged within 30 min at 1300 × g for 15 min at 4°C to obtain plasma samples. Blood drawn in vacutainer SST^{TM} II Advance tubes (Becton, Dickson and Company, Franklin Lanes, NY, United States) were first allowed to clot for at least 30 min at 21°C. These tubes were centrifuged at 1300 × g for 15 min at 21°C to obtain serum samples. Plasma and serum samples were immediately portioned into aliquots, frozen in liquid nitrogen, and stored at −80°C until analysis at the end of the study.

Plasma obtained from NaF tubes was used to determine glucose concentrations (Horiba ABX, Montpellier, France). Fasting serum samples were analyzed for insulin (RIA, Millipore, Billerica, MA, United States). The net incremental area under the curve (net iAUC) was calculated as described (Brouns et al., 2005). The homeostatic model assessment index was calculated as a measure of insulin resistance (HOMA-IR) (Matthews et al., 1985).

Cognitive Performance

Cognitive performance was assessed in silent chambers at FU-2 using the Cambridge neuropsychological test automated battery (CANTAB)[2]. These validated, computerized assessments

[2]https://www.cambridgecognition.com/cantab/

(Robbins et al., 1994, 1998; Louis et al., 1999) have been extensively described[2] before and measures performance in three cognitive domains: executive function, memory, and psychomotor speed. Based on the literature (Colcombe and Kramer, 2003; Angevaren et al., 2008; Guiney and Machado, 2013; Voss et al., 2013), the main hypothesis for the cognition parameters was that the reaction latency would improve following aerobic exercise training.

Executive function was assessed with the multitasking test (MTT) and spatial span (SSP). Focus was on four variables for the MTT: (1) incongruency cost (IC) was calculated by subtracting the median latency (ML) of response from the trials that were congruent from the incongruent trials; (2) multitasking cost (MTC) was determined as the difference between the ML of response, in which two rules were used (respond at the side the arrow appears or the direction the arrow points) compared to when only one of the rules was used; (3) ML of response for all correct trials; (4) The total number of errors (TE). For SSP, the maximal completed span length (SL) was used.

Memory was evaluated with the delayed matching to sample (DMS) and paired associates learning (PAL). The percentage of correctly answered trials for all delays (CAD) was used for DMS, while the first attempt memory score (FAMS) and TE were used for PAL.

Measurements of psychomotor speed included the motor screening task (MOT) and reaction time (RTI). The mean latency (LM) from target stimulus appearance to button press) outcome variable was used for MOT. For RTI the variables reaction time (RT) from target stimulus appearance to release of response button) and movement time (MT) from release of response button to selection of target stimulus) were used.

Statistical Analysis

Results are shown as mean ± standard deviation (SD), unless otherwise indicated. Before the start of the study, it was calculated that 15 participants were needed to reach a power of 80% to detect a true difference of 15% in CBF, which was the primary outcome parameter. For these calculations, a two-sided alpha of 0.05 and a within-subject variability of 19% were used (Gevers et al., 2011). CBF changes of 15% may be expected and are also clinically relevant (Alsop et al., 2000; Alexopoulos et al., 2012; Birdsill et al., 2013).

Intervention effects were examined using analysis of variances (ANOVA) with participant, treatment and period as fixed factors. Linear mixed models were performed to test for differences between treatments over time, using the change from baseline as dependent variable. Time, treatment, period and time * treatment interaction were used as fixed factors. If the interaction term was not statistically significant, it was omitted from the model. Bonferroni correction was used to correct for multiple comparisons. Statistical analyses were performed using SPSS (IBM Corp., IBM SPSS Statistics, V23, Armonk, NY, United States). Differences were considered statistically significant at $P < 0.05$ using two-tailed tests.

RESULTS

Study Participants

A CONSORT flow diagram of participants throughout the study is shown in **Figure 2**. In total 24 men were screened for eligibility. Five participants were excluded because they were not sedentary (two men), had an abnormal ECG (two men)

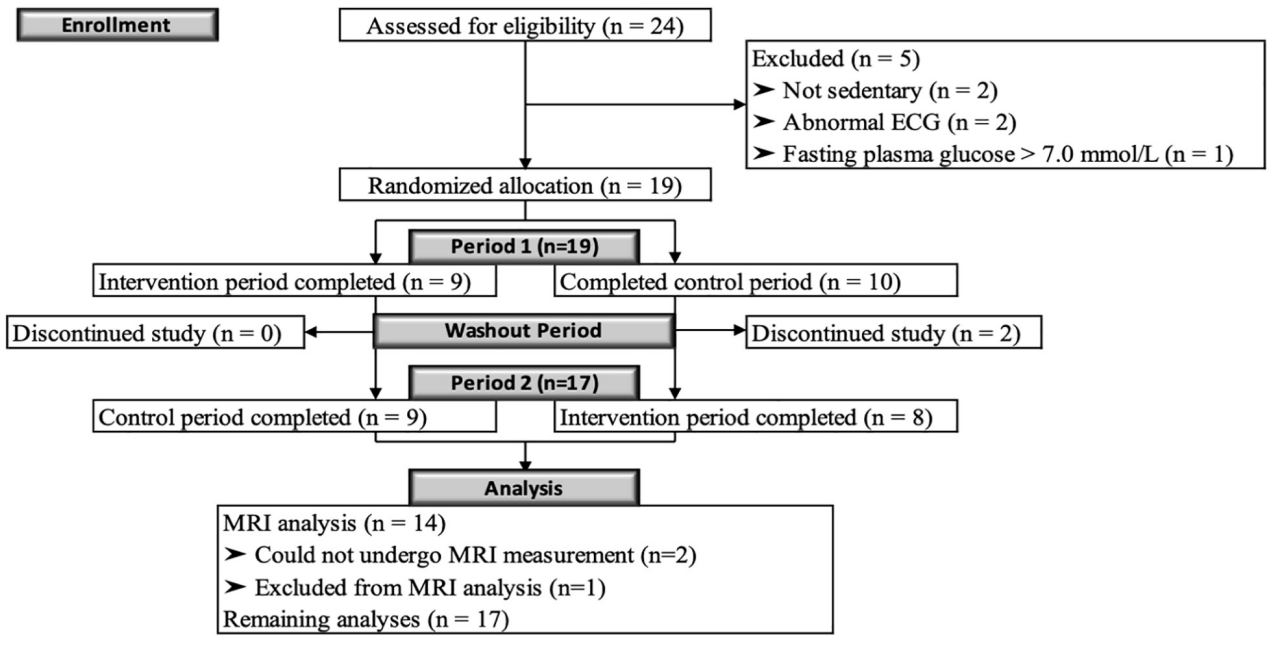

FIGURE 2 | CONSORT flow diagram. Diagram of the progress through the phases of this randomized, controlled crossover study with sedentary overweight or slightly obese older men.

or a fasting plasma glucose concentration above 7.0 mmol/L (one man). Thus, nineteen men were eligible and started the study. Two participants who started in the no-exercise control period dropped-out during the wash-out period for personal reasons and seventeen participants successfully completed the study. Two participants did not undergo the MRI measurements: one man became unexpectedly claustrophobic and another man due to remains of a metal screw in his skull following surgery which did not become apparent during the screening visit. Additionally, MRI data from one participant were excluded, because the magnetic field disturbance due to his tooth implant reduced the labeling efficiency of arterial blood. In total, fourteen participants were included in the final MRI analysis, while all seventeen participants were included in all other analyses. Baseline characteristics are shown in **Table 1**. Participants who completed the study had a mean age of 67 ± 2 years and a mean BMI of 30.3 ± 2.8 kg/m^2. Body weight remained stable at the follow-up measurements between both periods (0.9 ± 3.0 kg). The median attendance of the scheduled training sessions was 100% (range: 92 – 100%).

Maximal Exercise Test

All men reached a RER of at least 1.0 during all maximal exercise tests (1.12 ± 0.05), suggesting maximal exertion was reached. As expected, physical exercise training significantly increased aerobic fitness, as indicated by the significant time * treatment interaction for the VO_{2peak} (P = 0.018), using linear mixed models. Pairwise comparisons showed that the VO_{2peak} tended to increase by 99 ± 236 mL (P = 0.088) during the intervention period at week 4 and was significantly increased by 262 ± 236 mL (P < 0.001) at week 8 (**Figure 3A**). Comparable results were observed for P_{max} (time * treatment interaction: P < 0.001), which increased during the intervention at week 4 by 12 ± 18 W (P = 0.006) and at week 8 by 30 ± 18 W (P < 0.001; **Figure 3B**).

Cerebral Blood Flow

The mean CBF map from all participants after the control period is shown in **Figure 1B**. CBF differed between intervention and control periods in three clusters with a volume of 392 mm^3 (cluster 1), 616 mm^3 (cluster 2) and 408 mm^3 (cluster 3) (**Figure 4** and **Table 2**). CBF was increased by 28% (6.4 ± 5.0 mL/100 g

TABLE 1 | Baseline characteristics of sedentary older men who completed the study (n = 17).

Participant characteristics	
Age (y)	67 ± 2
BMI (kg/m^2)	30.3 ± 2.8
Total cholesterol (mmol/L)	5.28 ± 1.10
TAG (mmol/L)	1.39 ± 0.49
Glucose (mmol/L)	5.80 ± 0.36
Systolic blood pressure (mmHg)	138 ± 13
Diastolic blood pressure (mmHg)	88 ± 6

Data are shown as mean ± SD. BMI, body mass index; TAG, triacylglycerol.

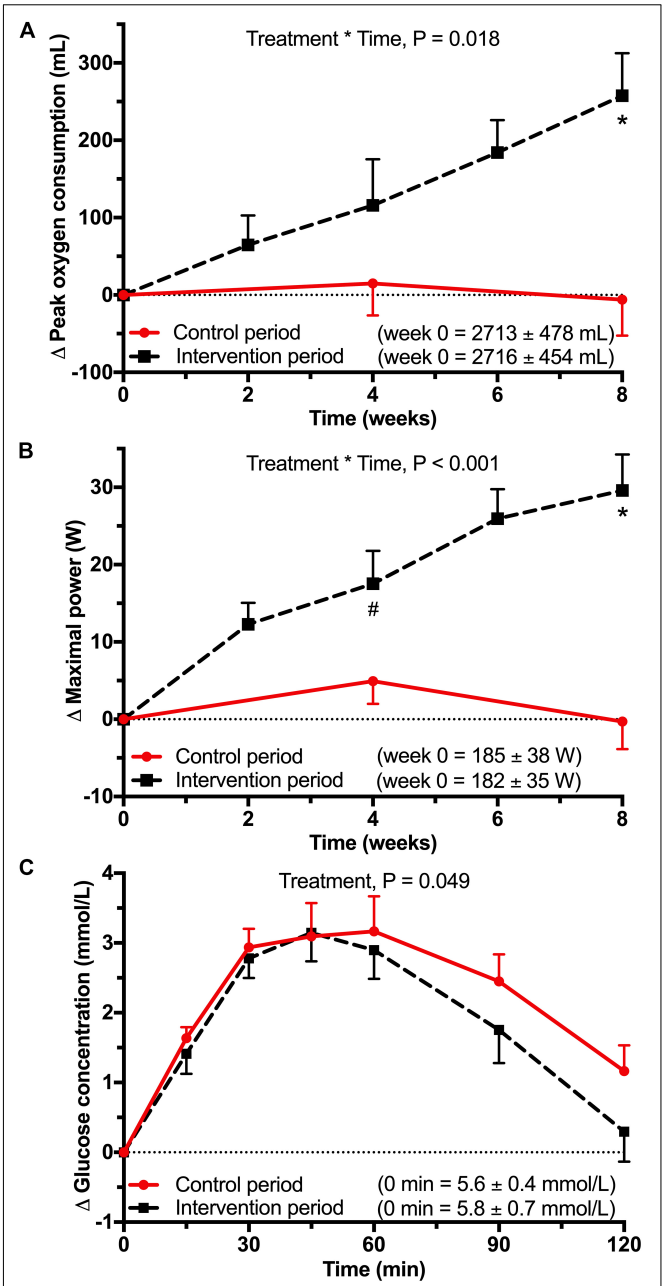

FIGURE 3 | Data from a randomized, controlled crossover study with sedentary overweight or slightly obese older men (n = 17). Data were analyzed using linear mixed models on the difference between each timepoint with baseline. **(A)** Mean (±SEM) difference in peak oxygen consumption (VO_{2peak}) and **(B)** maximal power (P_{max}) difference during the maximal exercise. Maximal exercise tests were performed every 2 weeks during the intervention period. During the control period, maximal exercise tests were performed at baseline, after 4 weeks and after 8 weeks. Baseline values were not significantly different. There was a significant treatment * time interaction for VO_{2peak} (P = 0.018) and P_{max} (P < 0.001). After Bonferroni correction there was a significant difference between control and intervention period at 4 weeks ($^{\#}P$ = 0.006) and at 8 weeks for VO_{2peak} and P_{max} ($^{*}P$ < 0.001). **(C)** Mean (±SEM) difference in glucose concentrations during a 7-point oral glucose tolerance test (OGTT) test. There was a significant treatment effect for glucose concentration (P = 0.049).

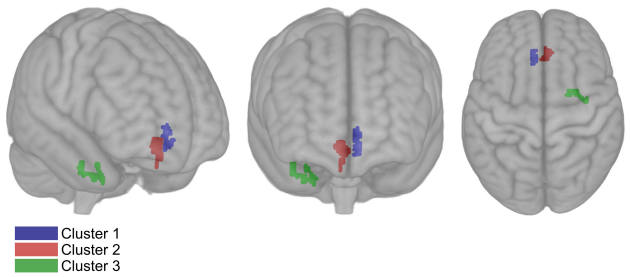

FIGURE 4 | Results of voxel-wise comparisons including all acquired cerebral blood flow (CBF) data in three dimensional MNI template from a randomized, controlled crossover study with sedentary overweight, or slightly obese older men (n = 14). CBF increased bilaterally after the intervention compared to the control period $P < 0.05$ (Family-wise error corrected). The CBF of cluster 1 and 2 increased after the intervention period compared to the control with 6.39 mL/100 g tissue/min (volume: 392 mm^3) and 6.95 mL/100 g tissue/min (volume: 616 mm^3), respectively. The CBF of cluster 3 decreased with 4.4 mL/100 g tissue/min (volume: 408 mm^3).

TABLE 2 | Mean ± SD cerebral blood flow difference between intervention and control period in a randomized, controlled crossover study with sedentary older men (n = 14).

	Intervention period (mL/100 g/min)	Control period (mL/100 g/min)	Mean difference (mL/100 g/min)	*P*
Gray matter CBF	27.6 ± 9.4	28.4 ± 7.9	−0.6 ± 3.6	0.533
Global CBF	23.5 ± 7.8	24.4 ± 6.4	−0.5 ± 3.0	0.523
Left hemi CBF	24.5 ± 8.7	25.4 ± 7.3	−0.5 ± 4.5	0.637
Right hemi CBF	25.7 ± 8.1	26.3 ± 6.7	−0.3 ± 3.9	0.723
Cluster 1 CBF	29.1 ± 12.2	22.7 ± 11.0	6.4 ± 4.8	0.040
Cluster 2 CBF	33.7 ± 17.8	27.0 ± 16.3	7.0 ± 4.9	0.001
Cluster 3 CBF	18.7 ± 5.3	23.1 ± 5.4	−4.4 ± 1.9	0.031

CBF, cerebral blood flow; hemi, hemisphere.

tissue/min; P = 0.040) in cluster 1 and by 26% (7.0 ± 4.8 mL/100 g tissue/min; P = 0.001) in cluster 2 after the intervention period. In contrast, CBF was decreased by 19% (−4.4 ± 1.9 mL/100 g tissue/min; P = 0.031) in cluster 3. The average probabilities for the locations of cluster 1 were 25% in the subcallosal cortex, 11% in the anterior cingulate gyrus, 8% in the paracingulate gyrus, and 3% in the frontal medial cortex. For cluster 2, which was located contralateral to cluster 1, the average probabilities were 24% in the subcallosal cortex, 23% in the frontal medial cortex, 15% in the paracingulate gyrus, and 12% in the anterior cingulate gyrus. For cluster 3, these probabilities were 35% in the temporal fusiform cortex and 25% in the parahippocampal gyrus.

We did not observe differences between the intervention and control periods (**Table 2**) in global CBF (−0.5 ± 3.0 mL/100 g tissue/min; P = 0.523), gray matter CBF (−0.6 ± 3.6 mL/100 g tissue/min; P = 0.533), CBF in the left hemisphere (−0.5 ± 4.5 mL/100 g tissue/min; P = 0.637), and CBF in the right hemisphere (−0.3 ± 3.9 mL/100 g tissue/min; P = 0.723).

Glucose Metabolism

A significant treatment effect was observed for the post-load glucose concentrations measured during the OGTT (P = 0.049) after the intervention period (**Figure 3C**). Pairwise comparisons showed a tendency toward lower glucose concentrations at 120 min (−0.86 ± 1.91 mmol/L; P = 0.083). Also, the net iAUC tended to decrease (−45 ± 96 mmol/L*2 h; P = 0.072).

Linear mixed models showed no time * treatment interactions for fasting glucose (P = 0.131) and insulin (P = 0.949) concentrations and the HOMA-IR (P = 0.772). There were also no significant treatment effects when this interaction term was omitted from the model (glucose: P = 0.146; insulin: P = 0.390; HOMA-IR: P = 0.423).

Cognitive Performance

Performance on the executive function MTT improved as indicated by a significant decrease in ML (−37 ± 65 ms; P = 0.034), while the number of total errors remained the same (0 ± 6; P = 0.770). The other MTT variables did not change. In addition, no changes were observed for the executive function test SSP, the memory tests DMS and PAL, and the psychomotor speed tests MOT and RTI (**Table 3**).

DISCUSSION

In this well-controlled, randomized trial in sedentary older men, aerobic exercise training affected regional CBF. It increased bilaterally in the subcallosal and anterior cingulate gyrus, which are both located in the frontal lobe. These two regions have been identified as important nodes in the limbic system and are involved in the regulation of executive cognitive functions (Vogt et al., 1992; Hamani et al., 2011). Reduced CBF was observed in one cluster

TABLE 3 | Mean ± SD between intervention and control period of cognitive outcomes in a randomized, controlled crossover study with sedentary older men (n = 17).

	Intervention period	Control period	Mean difference	*P*
MOT ML (ms)	846 ± 167	847 ± 177	−1 ± 157	0.980
RTI MT (ms)	281 ± 67	289 ± 56	−8 ± 56	0.574
RTI RT (ms)	399 ± 32	405 ± 45	−5 ± 33	0.510
MTT IC (ms)	117 ± 53	112 ± 48	4 ± 45	0.724
MTT MTC (ms)	281 ± 107	264 ± 109	17 ± 161	0.675
MTT RL (ms)	753 ± 87	790 ± 98	−37 ± 65	0.034
MTT IN	7 ± 9	7 ± 10	0 ± 6	0.770
SSP SL	6 ± 1	6 ± 1	0 ± 1	0.414
DMS TC (%)	80 ± 9	83 ± 7	−3 ± 12	0.336
PAL FAMS	11 ± 3	12 ± 2	−1 ± 2	0.282
PAL TE	17 ± 9	16 ± 9	2 ± 8	0.398

MOT, motor screening task; RTI, reaction time; MTT, multitasking test; SSP, spatial span; DMS, delayed matching to sample; PAL, paired associates leaning; LM, mean: latency; MT, movement time; RT, reaction time; IC, incongruency cost; MTC, multitasking cost; ML, median latency; TE, total errors; SL, forward span length; CAD, total correct; FAMS, first attempt memory score.

located in the right medial temporal lobe, mainly the temporal fusiform gyrus. In addition, latency of response was reduced for the executive function test and mean post-load glucose concentrations decreased. Recent findings suggest that unfavorable regional CBF alterations underlie reduced cognitive performance in older individuals, which may be mediated by impaired glucose metabolism (Bangen et al., 2018). This underlines the potential clinical relevance of the observed concomitant improvements in regional CBF, glucose metabolism, and executive function following exercise-induced increased fitness.

Cerebral blood flow increased by 27% bilaterally in the frontal lobe in clusters with a total volume of 1008 mm^3. The location of the cluster was comparable to the cluster identified by Chapman et al. (Chapman et al., 2013). However, they did not quantify the change in CBF, and the volume of the cluster was only 696 mm^3. Additionally, they did not show sustained increases in aerobic fitness. Therefore, the smaller cluster volume may be explained by the lower effectiveness of the intervention used. Reduced CBF in the frontal lobe is associated with increased age (Zhang et al., 2018), and a 4-year prospective longitudinal study observed that CBF at baseline was associated with cognitive performance at follow-up (De Vis et al., 2018).

Interestingly, CBF decreased by 19% in the right medial temporal lobe and this cluster had a volume of 408 mm^3. The observed decrease in CBF may attenuate progression of cognitive decline associated with human aging, as shown by a positive correlation of CBF in the temporal lobe with age (Preibisch et al., 2011; Zhang et al., 2018). Hays et al. (2018) have suggested that an increased temporal lobe CBF in a population with declined cognitive performance reflects neurovascular dysregulation. Additionally, increased CBF was observed in the medial temporal lobe early in the development of mild cognitive impairment (Dickerson and Sperling, 2008; Lacalle-Aurioles et al., 2014). In contrast to our findings, Maass et al. (2015) observed a decrease in hippocampal CBF after exercise in older individuals. Pereira et al. (2007) also showed an exercise-induced increase in cerebral blood volume in the dentate gyrus – a subregion of the hippocampus – in young and middle-aged participants. The intervention periods in these studies were 1 to 2 months longer than in our study, and image acquisition techniques were particularly optimized to detect changes in the hippocampus. This may have decreased the sensitivity to detect changes outside their region of interest, while our study may have been less sensitive to detect hippocampal changes due to coarser resolution and related partial volume effects. In addition, it is possible that longer intervention periods are needed to induce CBF changes in hippocampal brain regions. Indeed, Burdette et al. have shown that hippocampal CBF was higher following exercise training (Burdette et al., 2010). However, in this parallel study only post-intervention scans were performed, while the exercise training group consisted of 50% women compared to no women in the control group. Also, CBF was not corrected for hematocrit, which may have resulted in higher CBF values in women (Smith et al., 2019). Therefore, the observed CBF differences may be partly due to gender-mismatch instead of exercise training.

No changes in whole brain or gray matter CBF were observed. Gray matter CBF was based upon individually generated gray-matter masks in native space to ensure optimal overlap between structural gray matter regions and CBF. The mean gray-matter mask volume was comparable between the intervention and control periods within one participant (0.3 $\pm$ 3.0%). Gray matter CBF values were comparable with observed blood flow levels in studies that used partial volume correction (Preibisch et al., 2011; Bangen et al., 2018; Hays et al., 2018; Leeuwis et al., 2018) as incorporated in the FSL Basil tool. In fact, the mean gray matter CBF with partial volume correction in our study was 49.8 $\pm$ 13.0 ml/100 g tissue/min. However, we did not use this correction, also because the validity of proposed partial volume correction approaches has recently been questioned (Kirk et al., 2019). Decreased gray matter CBF has been observed in sedentary populations (Birdsill et al., 2013; Dai et al., 2017; Tarumi and Zhang, 2017), and was associated with accelerated cognitive decline (Wolters Frank et al., 2017). However, in agreement with our findings, other studies investigating the effect of aerobic exercise training on CBF also did not observe changes at the whole-brain level (Chapman et al., 2013; Maass et al., 2015).

Glucose metabolism improved, as indicated by the decreased post-load glucose concentrations. Besides the well-known effects of aerobic exercise training in (pre-)diabetics (Pan et al., 2018), Ferrara et al. (2006) also observed that exercise training increased glucose disposal in apparently healthy older men. Fasting plasma glucose concentrations did not change, which agrees with the result of a meta-analysis of 105 intervention studies (Boniol et al., 2017). The observed beneficial effects on CBF in specific brain regions may be linked to the improved glucose metabolism. This is supported by the relation between a reduced CBF in the frontal lobe and decreased cognitive performance in type 2 diabetic patients compared to healthy controls (Dai et al., 2017). Nevertheless, a causal relationship between peripheral and brain insulin sensitivity has not yet been established (Kullmann et al., 2015; Arnold et al., 2018).

Several reviews concluded that aerobic exercise training improves executive function (Colcombe and Kramer, 2003; Angevaren et al., 2008; Guiney and Machado, 2013; Voss et al., 2013), which is in line with the current findings. The latency of response decreased when the correct answer was given, while the number of errors remained unchanged, which indicates favorable effects on cognitive performance within the domain of executive function. This decrease in latency may be associated with improved response-inhibition, and was not due to speed-accuracy trade-off (Lo et al., 2015). The favorable effects on cognitive performance are in line with the improvements of CBF in the frontal lobe, which has been identified to be important in executive function (Yuan and Raz, 2014). No changes in cognitive performance in (visuo-spatial) memory or psychomotor speed were observed. Similarly, visuo-spatial memory did not improve following long-term aerobic exercise as shown in a meta-analysis of 21 aerobic exercise training

studies, whereas verbal-auditory memory did improve (Roig et al., 2013). Longer intervention periods may be needed to improve visuo-spatial memory, since only one trial with a 12-month physical training intervention showed beneficial effects on visuo-spatial memory (Erickson et al., 2011). Psychomotor speed only increased in studies with combined aerobic exercise- and resistance- or cognitive training (Levin et al., 2017). Multimodal combined training may thus be required to improve performance in psychomotor speed tests.

As expected, aerobic exercise training improved aerobic fitness during maximal exercise. VO_{2peak} increased significantly by 10% between the intervention and control group after 8 weeks, while P_{max} already increased after 4 weeks. These concomitant increases were expected based on the linear relationship between VO_{2peak} and P_{max} (Schoffelen et al., 2019). The consistent increase in VO_{2peak} and P_{max} of our trial emphasizes the effectiveness of the intervention. This may be attributed to a combination of several factors, including (i) the duration, frequency and tightly controlled supervised training sessions; (ii) the individually based progressive training intensity; and (iii) the inclusion of sedentary individuals. Maass et al. also showed an increase of 10% oxygen consumption at ventilatory anaerobic threshold after 12 weeks of 30 min interval training (Maass et al., 2015). In contrast, Chapman et al. only showed a change in VO_{2peak} at 6 weeks, which did not sustain after 12 weeks of aerobic exercise training (Chapman et al., 2013). Burdette et al. used a proxy-measure (400m walk speed) that did not significantly differ between groups and the duration and intensity of the home-based training sessions was not controlled (Burdette et al., 2010). The Train the Brain Consortium did not measure the effectiveness of the physical exercise training by means of an aerobic fitness outcome (Consortium TtB, 2017). Therefore, it cannot be assessed whether changes in CBF in these studies are due to exercise-induced changes in aerobic fitness.

Our tightly controlled, progressive, aerobic exercise training showed almost perfect attendance by the participants, generating consistent improvements in aerobic fitness across the 8-week intervention period. This trial included only men to reduce gender differences as an extra source of variability, which reduces the external validity. Additionally, although we were properly powered to find changes in our primary outcome, our sample size was too limited to examine into detail relationships between changes in aerobic fitness, CBF, glucose metabolism, and cognitive performance.

CONCLUSION

Our results show that aerobic exercise training improves regional CBF in sedentary older men. Also, cognitive performance in the domain of executive function improved, and beneficial effect on peripheral glucose metabolism were observed. Whether the observed exercise-induced changes in CBF underlie the beneficial effects on cognitive performance, and if they are mediated by changes in peripheral and/or brain insulin sensitivity requires further study.

DATA AVAILABILITY STATEMENT

All data supporting the conclusions of this study are presented in the article.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Ethics Committee of Maastricht University Medical Center (METC173025). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JK designed and conducted the study, performed the statistical analyses, interpreted the data, developed the analysis pipeline, and wrote the manuscript. RM and PJ designed the study, interpreted the data, had overall responsibility for the study, and wrote the manuscript. DI and KU developed the MRI sequences and analysis pipeline, interpreted the data, and reviewed the manuscript. JA developed the cognitive performance assessment protocol, interpreted the data, and reviewed 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.2019.00333/full#supplementary-material

FIGURE S1 | Schematic overview of study design. Timeline is displayed as weeks. FU-1, follow-up day one; FU-2, follow-up day two.

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frontiers in Aging Neuroscience

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Peripheral Maintenance of the Axis SIRT1-SIRT3 at Youth Level May Contribute to Brain Resilience in Middle-Aged Amateur Rugby Players

Rubén Corpas[1†], Elisabeth Solana[2†], Adrian De la Rosa[3†], Sara Sarroca[1], Christian Griñán-Ferré[4], Mireia Oriol[5], Emili Corbella[6], Eduard Rodríguez-Farré[1], Jose Vina[3], Mercè Pallàs[4], David Bartrés-Faz[2‡], Mari Carmen Gomez-Cabrera[3‡] and Coral Sanfeliu[1*‡]*

[1]*Institut d'Investigacions Biomèdiques de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Biomedical Research Centre Network for Epidemiology and Public Health (CIBERESP), Barcelona, Spain,* [2]*Departament de Medicina, Facultat de Medicina i Ciències de la Salut, Institut de Neurociències, Universitat de Barcelona, IDIBAPS, Barcelona, Spain,* [3]*Freshage Research Group, Department of Physiology, Faculty of Medicine, University of Valencia, Centro de Investigación Biomédica en Red Fragilidad y Envejecimiento Saludable (CIBERFES), Fundación Investigación Hospital Clínico Universitario/INCLIVA, Valencia, Spain,* [4]*Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia i Ciències de l'Alimentació, Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain,* [5]*Clinic Institute of Nephrology and Urology (ICNU), Hospital Clínic, Barcelona, Spain,* [6]*Unitat de Risc Vascular Medicina Interna, Hospital Universitari de Bellvitge, IDIBELL, CIBER Fisiopatología Obesidad y Nutrición (CIBEROBN), Barcelona, Spain*

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***Correspondence:**
Mari Carmen Gomez-Cabrera
carmen.gomez@uv.es
Coral Sanfeliu
coral.sanfeliu@iibb.csic.es

[†]These authors have contributed equally to this work

[‡]These authors have jointly supervised this work and share senior authorship

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Physical exercise performed regularly is known to improve health and to reduce the risk of age-related diseases. Furthermore, there is some evidence of cognitive improvement in physically active middle-aged and older adults. We hypothesized that long-term physically active middle-aged men may have developed brain resilience that can be detected with the analysis of peripheral blood markers. We aimed to analyze the activation of pathways potentially modulated by physical activity in a cohort of healthy amateur rugby players (n = 24) and control subjects with low physical activity (n = 25) aged 45–65 years. We had previously reported neuropsychological improvement in immediate memory responses in the player group compared to the controls. Here, we tested the expression of selected genes of longevity, inflammation, redox homeostasis, and trophic signaling in whole blood mRNA. Analyses were also performed on blood samples of young (aged 15–25 years) control subjects with low physical activity (n = 21). Physical activity and other lifestyle factors were thoroughly recorded with standardized questionnaires. Interestingly, middle-aged control subjects showed lower levels of expression of SIRT1, SIRT3, CAT, and SOD1 than the young controls, although rugby players maintained the expression levels of these genes at a young-like level. Middle-aged players showed lower levels of IL1B than the non-physically active groups. However, there was a tendency towards a decrease in trophic and transduction factors in middle-aged groups as compared to the young controls. A statistical study of Spearman's correlations supported a positive effect of sporting activity on memory

and executive functions, and on peripheral gene expression of SIRT1, SIRT3 and downstream genes, in the middle-aged rugby players. Our results indicate that the SIRT1-SIRT3 axis, and associated neuroprotective signaling, may contribute to the anti-aging resilience of the brain mediated by physical exercise.

Keywords: physical exercise, middle-aged and young men, whole-blood gene expression, SIRT1, SIRT3, brain resilience

INTRODUCTION

Health-promoting lifestyles have been proposed as a means of improving wellbeing, counteracting frailty and of delaying organismal aging and death. The combination of several healthy behaviors, that include a diet rich in fruits and vegetables, moderate alcohol consumption, non-smoking and regular physical activity, was reported to add a level of rejuvenation equivalent to 14 years in chronological age (Khaw et al., 2008). Similarly, lifestyles which include physical activity have proven to delay age-related cognitive impairment and decrease the risk of Alzheimer's disease (AD) and vascular dementia (Gallaway et al., 2017; Lin et al., 2019). Age-related processes of cell and tissue deterioration (López-Otín et al., 2013) are common in brain and peripheral organs. Furthermore, preservation of cell-survival mechanisms may induce milder AD phenotype (Sarroca et al., 2016). Conversely, frailty increases the risk of AD severity (Wallace et al., 2019). Therefore, we may speculate that healthy behaviors acquired early and maintained throughout a lifetime can build brain and body resilience against age-related ailments. Resilience, reserve, and resistance are concepts used in brain aging and AD to identify preventive interventions (Montine et al., 2019). The concepts of cognitive reserve and brain resilience similarly refer to the ability to maintain functional networks despite higher than expected brain pathological changes, whereas resistance refers to the ability of having lower than expected pathological changes (Negash et al., 2013; Arenaza-Urquijo and Vemuri, 2018). Furthermore, we hypothesized that resilience might be detected at early healthy aging when minor deterioration of the body and mind starts to show in subjects in their 50s.

In a recent study, we demonstrated that a cohort of middle-aged, amateur male rugby players showed physiological adaption to long-term physical activity. Namely, they showed lower resting levels in circulating brain-derived neurotrophic factor (BDNF) than aged-matched subjects with lower physical activity. Similar results were obtained from the simultaneous analyses of young men who regularly practiced sports vs. age-matched sedentary men (De la Rosa et al., 2019). Interestingly, the veteran rugby players scored slightly but significantly higher statistically than the age-matched controls in neuropsychological tests of declarative verbal memory (De la Rosa et al., 2019). Therefore, this middle-aged collective could help to discern differential markers of early cognitive aging linked to physical activity-induced resilience. Exercise training activates transcriptional regulators and enzymes to reprogram many pathways in multiple adaptive organismal responses. We aimed to analyze peripheral blood samples for changes in gene expression in the main pathways related to physical activity as described below:

(a) Physical exercise activates cell mechanisms, regulating energy expenditure that increases nicotinamide adenine dinucleotide (NAD^+) levels in response to the higher intracellular AMP/ATP ratio (Cantó et al., 2009). Increases in NAD^+ will induce the activation of the NAD^+-dependent histone deacetylase sirtuin family of proteins that improves healthspan (Haigis and Sinclair, 2010). Sirtuins (SIRT1-SIRT7) deacetylate, and therefore activate, many enzymes and transcription factors crucial for maintaining cell physiology, stress response, and survival signaling. The sirtuins examined in this study, SIRT1, SIRT2, SIRT3, and SIRT6, are potential targets in the fight against brain aging and neurodegeneration (Satoh et al., 2017). SIRT1 and SIRT6 are preferentially located in the nucleus, SIRT3 in mitochondria and SIRT2 in the cytosol; all of them are expressed in a variety of body tissues and organs including the brain, blood cells and skeletal muscle (Grabowska et al., 2017).

(b) Increased oxidative damage is one hallmark of tissue and organ aging. It has long been known that antioxidant enzymes are up-regulated by physical exercise as a hormetic response to bursts of oxidative stress caused by increased metabolic activity (Radak et al., 2008). Some reports show discrepancies as to whether such enzymes vary with aging or that such aging is tissue-dependent (Zhang et al., 2015). However, some decreases might contribute to age-associated oxidative stress. Habitually doing physical exercise at an old age may prevent the establishment of age-related oxidative stress (Pierce et al., 2011) and reduce immunosenescence features (Duggal et al., 2018). We analyzed the genes CAT, SOD1, SOD2, GPX1 and GPX4, codifying for catalase, Cu/Zn superoxide dismutase, Mn superoxide dismutase, and glutathione peroxidase isoforms 1 and 4, respectively. The gene NFE2L2 codes for the transduction factor Nuclear factor (erythroid-derived 2)-like 2 (i.e., Nrf2), that regulates the expression of detoxification and antioxidant genes. Sirtuins may activate the expression of antioxidant genes through the deacetylation of Nrf2 (Singh et al., 2018). Additionally, SIRT1 may promote the expression of SOD2, CAT, and other antioxidant genes through the deacetylation of FOXO3a and PGC-1α transcription factors (Olmos et al., 2013). Furthermore, SIRT3 post-translationally activates SOD2 and other mitochondrial enzymes in response to the presence of reactive oxygen species (Chen et al., 2011).

(c) There is chronic low-grade systemic inflammation in aging that contributes to the risk of age-related illnesses and

memory loss (Bradburn et al., 2018; Rea et al., 2018). Regular exercise downregulates several pro-inflammatory pathways in the elderly (Woods et al., 2012). We analyzed gene expression of the pro-inflammatory cytokines interleukin 1β (IL1B) and interleukin 6 (IL6) and the anti-inflammatory cytokine IL10, all widely characterized in health and disease. SIRT1 may directly inactivate NF-kB; SIRT6 may do it indirectly (i.e., through intermediate processes; Yeung et al., 2004; Kawahara et al., 2009). Therefore, sirtuins may contribute to decreasing inflammation. Furthermore, changes in redox homeostasis may regulate inflammatory genes (Lavrovsky et al., 2000).

(d) Lastly, physical activity promotes trophic factor signaling (Cobianchi et al., 2017). Myokines such cathepsin B (CTSB; Moon et al., 2016), vascular factors such as vascular endothelium growth factor A (VEGFA) and other peripheral factors induced by exercise may have a positive impact on brain tissue. One of the best characterized is the BDNF also synthesized in the brain (Marosi and Mattson, 2014). The cAMP response element-binding protein 1 (CREB1) is a major mediator of neurotrophin responses.

All of these genes are expressed in blood cells and we analyzed their levels in middle-aged amateur rugby players and age-matched controls, adding a group of young subjects as age control. Subsequently, we searched for correlations in gene expression with the previously reported changes in declarative verbal memory (De la Rosa et al., 2019) and with other neuropsychological data that did not showed significant changes between both middle-aged groups. We also searched for correlations with behavior potentially related to health and resilience. For this purpose, we examined physical activity and other lifestyles and health behaviors through standardized questionnaires. We suggest that these analyses may help to discern genes or gene pathways involved in the promotion of brain resilience.

MATERIALS AND METHODS

Study Design and Participants

Middle-aged amateur rugby players participating in veterans' competitions (males, $N = 24$), age-matched controls with low physical activity (males, $N = 25$) and young controls with low physical activity (males, $N = 21$) were enrolled in a study approved by the Ethics Committee of the Hospital Clínic de Barcelona, Spain (Reg. HCB/2014/0759). Written informed consent was obtained from all participants in the study in accordance with the Declaration of Helsinki. The rugby players were long-term practitioners of the sport, engaged in weekly training and game playing for an average period of 35 years (range: 7–59 years). Control subjects did either no sports or leisure physical activity at all, or they performed such activities for less than 150 min per week, as recorded in the STEPS questionnaire (see below). Reasons for exclusion from the study included: consumption of neuroactive drugs, medical histories of brain disease or other serious health conditions or moderate or severe traumatic brain injury. Mild traumatic brain injuries, namely concussions, were recorded. Subjects were recruited during 2014 and 2015. Neuropsychological testing and blood sampling were performed in 2015. A previous study with this cohort is reported in De la Rosa et al. (2019).

All subjects were interviewed by a neuropsychologist trained in clinical studies and were also asked to fill out self-administered questionnaires. Middle-aged subjects were tested with a battery of neuropsychological tests. All tests and questionnaires were validated and recognized in the field. Blood samples were obtained by a trained nurse, processed and stored at −80°C until use. The support of the Mario Sàlvia Foundation of the Institut d'Estudis Catalans allowed the Cerebral Aging and Lifestyles (*Envelliment Cerebral i Estils de Vida*, ECEV) sample collection to be established in the National Registry of Biobanks, from the Instituto de Salud Carlos III, Spain.

Physical Activity and Lifestyle Questionnaires

All subjects of the study were asked to fill out the following questionnaires:

(a) WHO STEPwise approach to chronic disease risk factor surveillance (STEPS) questionnaire, core information. Information to be filled out includes the health variables of hypertension, hyperglycemia, body height, and weight, smoking habits, diet habits (including alcohol consumption), and physical activity habits (WHO STEPS Surveillance Manual, 2008). Degrees of physical activity was given as hours spent doing the activity in a representative week.

(b) International Physical Activity Questionnaire (IPAQ), long version. Information on the health-related physical activity refers to the last 7 days (Craig et al., 2003). Results were calculated as the weekly metabolic equivalent of a task in minutes (MET-min) for the different categories of physical activity.

(c) Minnesota Leisure-Time Physical Activity Questionnaire (MLTPAQ) quantifies the metabolic rate of different leisure physical activities performed during the last 12 months (Folsom et al., 1986). Values were calculated as the yearly total MET-h.

Psychological Questionnaires

All subjects of the study were asked to fill out the following scales and questionnaires:

(a) Hamilton Rating Scale for Anxiety (Hamilton-A). Rates 14 groups of symptoms of anxiety, on a scale of 0–5 points. Composite scores of 0–17 indicate absence of or mild anxiety; 18–24 indicate mild to moderate anxiety; and 24–30 indicate moderate to severe anxiety (Hamilton, 1959).

(b) Hamilton Rating Scale for Depression (Hamilton-D). Rates 17 symptoms of depression on a scale of 0 and 3 points. Scores of 0–7 indicate the absence of depression and 20 or higher indicate at least moderate depression (Hamilton, 1960).

(c) Pittsburgh Sleep Quality Index (PSQI). Rates seven components of sleep quality on a scale of 0–3. The overall score ranges from 0 to 21, with low scores of 0–5 indicating good sleep quality (Buysse et al., 1989).

(d) Memory Functioning Questionnaire (MFQ). A 7-item scale, with each item rated from 1 to 7, including different aspects of self-appraisal of memory, forgetfulness and mnemonics usage. Higher composite scores indicate better self-appraised memory functioning (Gilewski et al., 1990).
(e) Cognitive Reserve Variables Questionnaire. Gives a composite score for the presence of lifestyles considered to increase cognitive reserve including education, occupational work and leisure activities of intellectual, social and physical activity type (Solé-Padullés et al., 2009). In the validation of this cognitive reserve score, the authors reported that cognitively-healthy adults older than 65 years scored 7–11, whereas cognitively-impaired subjects scored 3–7 (Solé-Padullés et al., 2009).

Blood Collection and Extraction of DNA and RNA Samples

Peripheral blood samples from all the subjects were obtained from the antecubital vein following overnight fasting. Blood was collected in different tubes in accordance with the required testing methods. BD Vacutainer™ tubes (BD Diagnostics, Frankin Lanes, NJ, USA) containing anticoagulant EDTA were used for DNA analysis. Once the blood samples were collected, the tubes were gently inverted 8–10 times and then frozen at −80°C until DNA extraction. The DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA) in accordance with the manufacturer's instructions. Tempus™ Blood RNA tubes (Thermo Fisher Scientific, Waltham, MA, USA) were used for the stabilization of total RNA for gene expression analysis. Once the blood samples were collected, the tubes were shaken vigorously for 10 s and frozen at −80°C until RNA extraction. Extraction was performed using the *mir*Vana™ miRNA Isolation Kit with phenol (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions for obtaining total RNA, including small RNA. Purity and concentration of DNA or RNA were assessed in a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific). Blood samples were collected the same day or within a close time frame of the neuropsychological testing for the middle-aged subjects (see below).

Genetic Analysis

DNA samples were analyzed to determine APOE allele distribution. The APOE gene is polymorphic at two single nucleotides (rs429358 and rs7412), resulting in the alleles ε2, ε3, and ε4. APOE ε4 is considered a risk factor for various conditions associated with a cognitive loss with advancing age. It was analyzed with the quantitative Polymerase Chain Reaction (qPCR), using a combination of two TaqMan probes doubly marked with 6-carboxyfluorescein (FAM) and VIC fluorescents (Taqman SNP Genotyping Assays, Applied Biosystems), which demonstrated the different combinations of the alleles ε2, ε3 and ε4 (Zhong et al., 2016).

RNA samples were analyzed to quantify the expression of selected genes. Genes of the sirtuin family of proteins involved in longevity and neuroprotection (SIRT1, SIRT2, SIRT3 and SIRT6), antioxidant enzymes and related factors (CAT, SOD1, SOD2, GPX1, GPX4 and NFE2L2), inflammatory-related proteins (IL1B, IL6 and IL10), and trophic factors and downstream effectors (BDNF, NTRK2, CREB1, CTSB and VEGFA). For qPCR analysis, random-primed cDNA synthesis was performed using the High-Capacity cDNA Archive kit (Applied Biosystems). Gene expression was measured with specific TaqMan FAM-labeled probes (Applied Biosystems) in a CFX96 Real-Time qPCR Detection System (Bio-Rad, Hercules, CA, USA). Data were normalized to PGK1 and B2M. Results were calculated using the 2-ΔΔCt method and expressed relative to the middle-aged control group. A list of probes utilized is presented in **Supplementary Table S1**.

Analysis of Superoxide Dismutase Enzymatic Activity and Interleukin 1β Protein Levels

Enzymatic activity of SOD was selected as a measure of antioxidant changes in plasma. The analysis was performed using a 19160 SOD Determination Kit (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. The SOD activity was calculated as the inhibition rate of the reduction of a water-soluble formazan dye by superoxide anion.

The protein level of IL1B was determined as a representative pro-inflammatory cytokine in plasma using a Quantikine HS ELISA Kit (#HSLB00D; R&D Systems, Minneapolis, MN, USA).

Neuropsychological Tests

Subjects of both middle-aged groups; controls and rugby players, were submitted to the following neuropsychological tests:

(a) Free and Cued Selective Reminding Test (FCSRT). In this test, declarative learning and memory are evaluated using a list of 16 words, which must be remembered over the course of three attempts. Both immediate and long-term memory is evaluated freely and with the help of semantic cues (Buschke, 1984). Scores are given for immediate free recall, immediate cued recall, delayed free recall and delayed cued recall. See a detailed description of the test in De la Rosa et al. (2019).
(b) Trail Making Test (TMT). In the TMT Part A, a series of numbers printed on a sheet of paper must be connected using a pen-drawn line from the smallest to the largest and at the highest possible speed. This subtest evaluates psychomotor speed and attention. In the TMT Part B, numbers and intercalated letters should be joined in numerical order and alphabetical order (Llinàs-Reglà et al., 2017). This subtest also evaluates a component of executive functions. Shorter time in seconds to complete either TMT-A or TMT-B indicates a better response.
(c) Symbol Digit Modality Test (SDMT). This test measures psychomotor speed and attention through a substitution task where geometric symbols, repeatedly listed at random, must be paired with the corresponding numbers from 1 to 9. The score is the number of correct substitutions performed in 90 s (Smith, 1982).
(d) Wechsler Adult Intelligence Scale fourth edition (WAIS-IV). The WAIS test was designed to measure intelligence and

cognitive abilities in clinical practice (Wechsler, 1955). Here we used several subtests of the current version WAIS-IV. The direct digits subtest evaluates attention and working memory, the inverse digits subtest evaluates executive functions, working memory and vocabulary, as well as premorbid intellectual level.

(e) Verbal fluency. Executive functions are also assessed by verbal fluency tasks where the participants must issue as many words as possible of a given semantic category or that begin with a given letter, for 1 min (Lezak et al., 2012). The score is the number of words issued.

(f) Stroop color and word test. The automatic task of reading a list of words of color names interferes with the requested task of naming the ink color of the words; the phenomenon is known as the Stroop effect. A score of correct hits measures processing speed and the executive function of inhibition (Golden, 1978).

(g) Cambridge Neuropsychological Test Automated Battery (CANTAB). The CANTAB tests are computerized programs designed to assess several cognitive areas (Robbins et al., 1994). Here we used Spatial Working Memory (SWM), Paired Association Learning (PAL) and Rapid Visual Information Processing (RPV) tests.

Statistics

Results are displayed as mean ± SD for normally distributed variables, median [interquartile range (IQR)] for non-normal variables and number (percentage) for qualitative variables. The Normality of distribution for quantitative variables was analyzed by the Shapiro–Wilk test. For comparisons between three groups, quantitative normal variables were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons, and non-normal variables were analyzed by the Kruskal–Wallis test followed by Dunn's test. Data pairs were analyzed using the two-tailed Student's *t*-test or Mann–Whitney test as appropriate. Qualitative variables were analyzed using the Chi-square test. Bivariate correlations were analyzed with the two-tailed Spearman's rho. Statistical analyses were performed using the GraphPad Prism v 6.01 (GraphPad, La Jolla, CA, USA) and the IBM Statistical Package for the Social Sciences (SPSS) software v 23.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Characteristics of the Study Groups

Sociodemographic variables, selected habits and health parameters of the subjects enrolled in the study are shown in **Table 1**. Both middle-aged groups had a similar mean outcome in age and years of scholarship. Subjects were typically in their mid-fifties, with a mean age of 56.0 for the control group; including subjects from 47 to 67 years and a mean age of 54.3 years for the rugby players; including subjects from 46 to 68 years. The young control group showed a mean age of 20.9 years with subjects ranging from 17 to 24 years; some of them have not finished their years of scholarship and this parameter is not considered for this group. However, they had at the time of testing an average education level similar to that of the other groups, between high school and university level (not shown). Body mass index (BMI) and occurrences of hypertension and hyperglycemia were similar among middle-aged groups. The young subject group showed a lower BMI and lower incidences of hypertension. The frequency of APOE allele ε4 was similar among all groups. No subject presented the form ε4 for both alleles of the gene. Frequency of all three APOE alleles and the resulting genotypes (not shown) was in agreement with the general distribution in the population (Corbo and Scacchi, 1999). The rugby player group showed higher incidences of concussion than the age-matched control group. Habits of smoking and diet were similar among groups, albeit with a slightly lower fruit intake in young controls. The levels of anxiety, depression, and quality of sleep (as analyzed with the self-administered questionnaires Hamilton-A, Hamilton-D, PSQI, and MFQ, respectively) of all subjects assessed were within the normal range. No differences were detected among the study groups, but a lower subjective memory function of young controls in the MFQ. In the questionnaire of Cognitive Reserve Variables, all groups showed normal mean scores, and the significantly higher value of the rugby players is probably a reflection of their higher physical activity component.

Physical Activity

The veteran rugby players performed significantly higher rates of physical activity during leisure time than the age-matched controls and young controls, as displayed in **Table 2**. The statistical differences were consistent in the outcome of the three questionnaires: IPAQ, STEPS, and MLTPAQ. The player group reported significantly higher values of physical activity during leisure than middle-aged and young control groups in the 1-week IPAQ questionnaire. Physical activity at work or at home and in the garden did not differ among the groups. No differences in walking or bicycling were also detected. Nevertheless, the IPAQ total MET remained higher in the player group as compared to both control groups showing median values of MET-min/week of two times the middle-aged control group and 3.2 times the young control group. Similarly, in the STEPS questionnaire, there were no differences in physical activity at work or in travel, but in leisure activities. The greatest difference between both control groups and the player group was found in the intense physical activity performed during leisure. In the 1-year records of all leisure activities in the MLTPAQ, the player group showed median values of MET-h/year of 2.2 times the middle-aged control group and 1.8 times the young control group. Rugby accounted for a third of all leisure activities of the player group in total MET.

Gene Expression in Peripheral Whole Blood

Genes of the sirtuin family SIRT3 and SIRT1 and some downstream-related genes showed differential expression between middle-aged rugby players and middle-aged controls. Expression levels of genes SIRT3 and SIRT1, antioxidant genes CAT and SOD1 and pro-inflammatory gene IL1B are shown in graphs **Figures 1A–E**, respectively. The middle-aged control group showed significant lower

TABLE 1 | Sociodemographic, dietary habits and health status parameters.

	Middle-aged controls *N* = 25	Middle-aged rugby players *N* = 24	Young controls *N* = 21	*P*
Sociodemographic variables				
Age (years)	56.0 ± 5.9	54.3 ± 6.6	20.9 ± 2.2***,###	<0.0001
Schooling (years)	14.4 ± 3.3	14.9 ± 3.4	ND	0.5885
Clinical and genetic variables				
Body mass index (Kg/m^2)	28.4 ± 3.9	28.9 ± 2.7	24.2 ± 4.6**,###	0.0001
Hypertension				
Diagnosis (*N*, %)	10 (40%)	10 (41.7%)	1 (4.8%)**,##	0.0105
Treatment (*N*, %)	5 (20%)	5 (20.8%)	0 (0%)	0.0818
Hyperglycemia				
Diagnosis (*N*, %)	5 (20%)	3 (12.5%)	0 (0%)	0.1027
Treatment (*N*, %)	2 (8%)	1 (4.2%)	0 (0%)	0.4103
APOE ε4 (*N*, %)[1]	7 (28%)	5 (21%)	4 (19%)	0.7395
Concussion[2] (*N*, %)	4 (16%)	12 (50%)*	ND	0.0157
Smoking and dietary habits				
Tobacco use (*N*, %)				
Never	9 (36%)	12 (50%)	13 (62%)	
Former	8 (32%)	8 (33.3%)	1 (4.8%)	0.0994
Current	8 (32%)	4 (16.7%)	7 (33%)	
Fruit consumption (servings/week)	12 (7–21)	5 (3–14)	3 (2–14)*	0.0362
Vegetable consumption (servings/week)	6 (4–14)	5.5 (4–8.25)	8 (4–14)	0.6481
Alcohol drinking frequency[3]	3 (2–5)	3 (2–4.75)	2 (1–3)	0.1680
Psychological variables				
Hamilton rating scale for anxiety	8.1 ± 1.9	8.0 ± 2.0	9.4 ± 3.2	0.0926
Hamilton rating scale for depression	8.4 ± 1.4	9.1 ± 1.4	8.4 ± 2.5	0.3037
Pittsburgh sleep quality index	5.3 ± 2.6	4.7 ± 2.2	5.6 ± 1.8	0.4175
Memory functioning Q	93.8 ± 10.3	91.7 ± 13.6	84.8 ± 12.3*	0.0466
Cognitive reserve variables Q	13.8 ± 3.0	16.8 ± 3.3*	14.2 ± 2.9##	0.0021

*Data are presented as mean ± SD, median (IQR), or number (%). Statistics: ANOVA or Kruskal–Wallis test, followed by Tukey's test or Dunn's test, respectively, Chi squared test, or Student's t-test, where appropriated; *P < 0.05, **P < 0.01, ***P < 0.001 compared to Middle-aged control group, ##P < 0.01, ###P < 0.01 compared to Middle-aged rugby player group. ND, not determined. NOTES: [1]subjects with one allele (no subjects with two alleles were detected; [2]subjects with at least one episode; [3]alcohol drinking last year: from 0, no drinking, to 5, daily intake.*

TABLE 2 | Physical activity parameters.

	Middle-aged controls *N* = 25	Middle-aged rugby players *N* = 24	Young controls *N* = 21	*P*
IPAQ (MET-min/week)				
PA at work	30 (0–1,091)	0 (0–3,990)	0 (0–0)	0.2258
PA in travel, walking and bicycling	363 (181–990)	477 (132–864)	600 (330–10,539)	0.5057
PA at home or in garden	885 (0–1,541)	420 (0–1,905)	175 (80–630)	0.5738
PA during leisure	198 (0–531)	3,078 (1,440–4,386)***	0 (0–396)###	<0.0001
Total PA	2,298 (1,604–3,846)	4,606 (2,962–10,647)*	1,435 (990–1,886)###	<0.0001
STEPS, PA (min/week)				
Moderate PA at work	0 (0–50)	0 (0–0)	0 (0–0)	0.2944
Intense PA at work	0 (0–0)	0 (0–0)	0 (0–0)	0.3585
PA during walking and bicycling	22 (0–162)	0 (0–124)	100 (30–240)	0.1513
Moderate PA during leisure	0 (0–90)	90 (0–240)	0 (0–15)##	0.0089
Intense PA during leisure	0 (0–0)	240 (90–450)***	0 (0–0)###	<0.0001
MLTPAQ (MET-h/year)				
All leisure activities	1,379 (751–4,702)	3,081 (2,209–5,232)**	1,709 (935–2,583)##	0.0022
Rugby	-	742 (546–1181)	-	

*Data are presented as median (IQR). Statistics: Kruskal–Wallis test, followed by Dunn's test; *P < 0.05, **P < 0.01, ***P < 0.001 compared to Middle-aged control group, ##P < 0.01, ###P < 0.001 compared to Middle-aged rugby player group. Abbreviations: PA, physical activity; IPAQ, International Physical Activity Questionnaire; STEPS, WHO STEPwise Approach to Chronic Disease Risk Factor Surveillance questionnaire; MLTPAQ, Minnesota Leisure Time Physical Activity Questionnaire.*

expression of SIRT3, SIRT1, CAT and SOD1 than the young controls. Strikingly, the middle-aged rugby players maintained SIRT3, SIRT1, CAT and SOD1 expression at the same level as the young controls. The difference between both middle-aged groups was statistically significant for SIRT3, CAT and SOD1 and of borderline significance for SIRT1 ($P = 0.064$). Furthermore, middle-aged players showed statistically significant lower expression of IL1B than the other two groups.

The results of analyzed genes that did not show differences in expression between both middle-aged groups are shown in **Figure 2**. Expression of pro-inflammatory cytokine

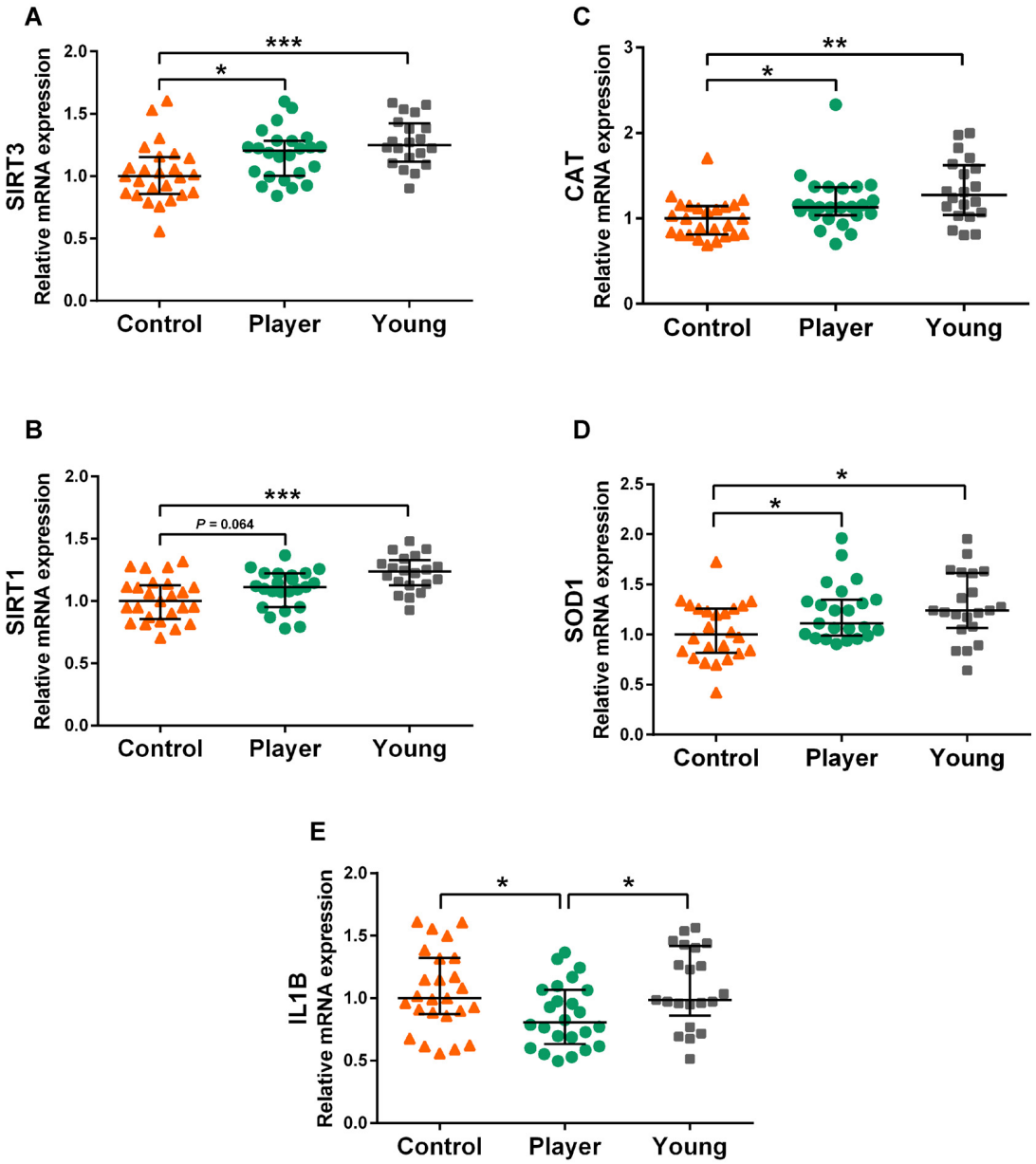

FIGURE 1 | Gene expression levels of SIRT3 **(A)**, SIRT1 **(B)**, CAT **(C)**, SOD1 **(D)**, IL1B **(E)** in whole blood mRNA. Data are presented as median [interquartile range (IQR)]. Statistics: Kruskal–Wallis followed by Dunn's test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

IL6, anti-inflammatory cytokine IL10, sirtuins SIRT2 and SIRT6, antioxidants SOD2 and GPX1 are displayed in graphs **Figures 2A–F**, respectively. No differences in expression among the three study groups were detected for these genes. Expression of the antioxidant-related genes GPX4 and NFE2L2 and tropism-related genes BDNF, NTRK2, CREB1, CTSB, and VEGFA are displayed in graphs **Figures 2G–M**, respectively. Both middle-aged groups showed lower expression of GPX4 and NFE2L2 than the young controls. The middle-aged control group showed lower expression in the signaling factor CREB1 than the young controls. The player group showed a lower expression in BDNF and VEGFA than the young controls. Indeed, the trophic factor genes BDNF, CTSB and VEGFA showed a tendency to decrease in both middle-age groups. No differences among groups were detected for the BDNF receptor gene NTRK2.

Superoxide Dismutase Enzymatic Activity and Interleukin 1β Protein Levels in Plasma

Decreased SOD1 gene expression in whole blood of middle-aged control group did not induce a significant decrease in SOD enzymatic activity in the corresponding plasma samples, but

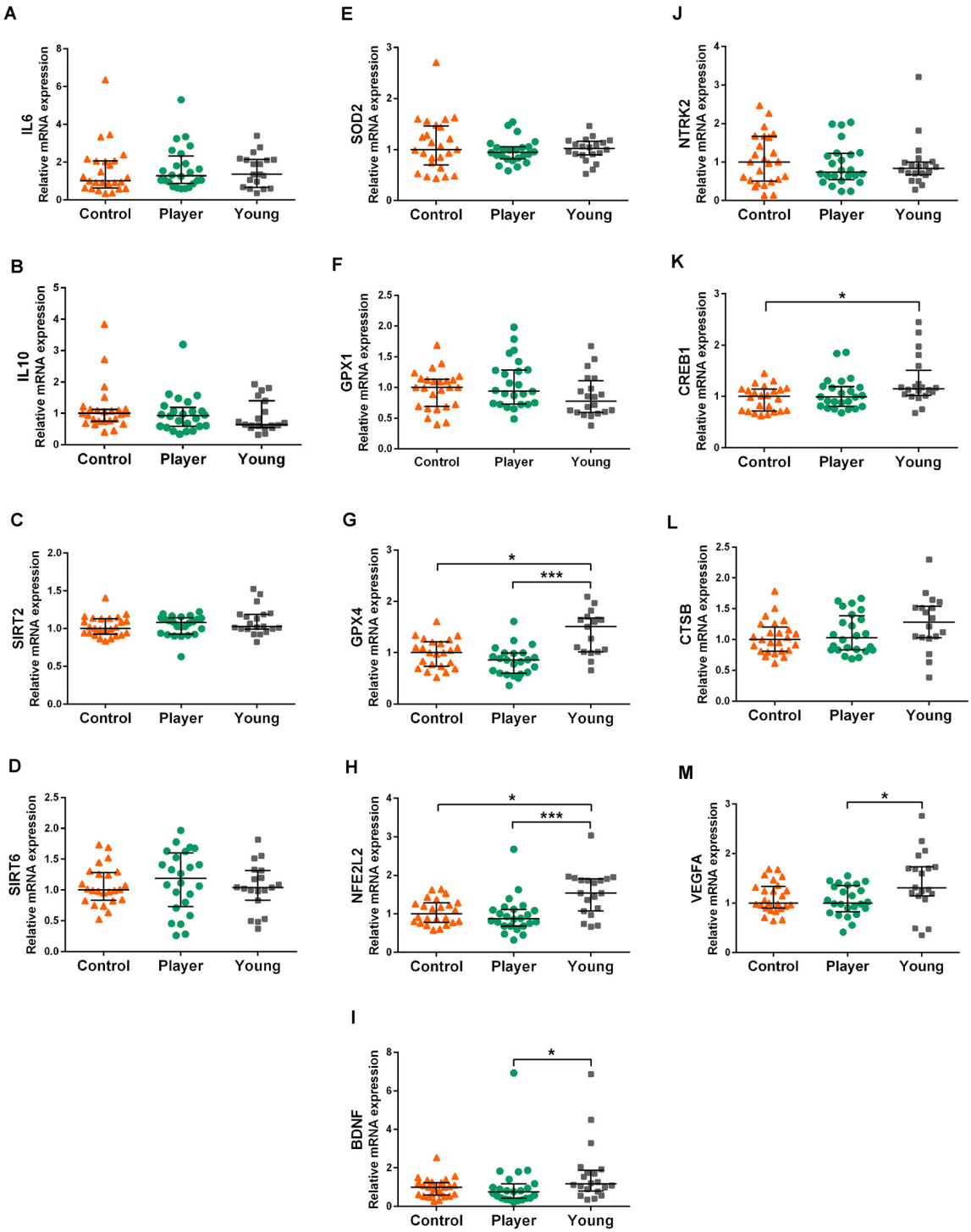

FIGURE 2 | Gene expression levels of IL6 **(A)**, IL10 **(B)** SIRT2 **(C)**, SIRT6 **(D)**, SOD2 **(E)**, GPX1 **(F)**, GPX4 **(G)**, NFE2L2 **(H)** BDNF **(I)**, NTRK2 **(J)**, CREB1 **(K)**, CTSB **(L)** and VEGFA **(M)** in whole blood mRNA. Data are presented as median (IQR). Statistics: Kruskal–Wallis followed by Dunn's test, $^{*}P < 0.05$, $^{***}P < 0.001$.

only a minor trend. Results of SOD activity for Control, Player and Young groups were respectively: 66.4 (62.5,69.7), 67.7 (64.2,70.6) and 68.3 (65.8,70.5), expressed as median (IQR). The Kruskal–Wallis test was not significant.

Changes of IL1B protein levels showed borderline significant changes suggesting higher levels of this cytokine in middle-aged controls. Results for Control, Player and Young groups were respectively: 0.72 (0.13,2.64), 0.34 (0.06,0.88) and 0.07

TABLE 3A | Neuropsychological testing.

	Middle-aged controls *N* = 25	Middle-aged rugby players *N* = 24	*P*
Declarative memory			
FCSRT, Immediate free recall[1]	18.2 ± 5.1	21.8 ± 5.9*	0.0270
FCSRT, Immediate cued recall[1]	27.3 ± 5.1	31.1 ± 5.2*	0.0123
FCSRT, Delayed free recall	8.3 ± 2.2	9.3 ± 2.8	0.1662
FCSRT, Delayed cued recall	12.1 ± 2.5	13.2 ± 2.3	0.1318
Attention and psychomotor speed			
TMT, Part A	27.2 ± 7.5	25.8 ± 6.1	0.4646
SDMT	48.4 ± 8.8	51. 7 ± 8.7	0.2023
WAIS IV, Direct digits	9.2 ± 1.9	10.0 ± 2.4	0.2194
Executive functions			
TMT, Part B	68.0 ± 19.1	58.4 ± 18.0	0.0781
WAIS IV, Inverse digits	6.4 ± 2.7	6. 7 ± 2.4	0.6745
Verbal fluency, Semantic	20.8 ± 4.7	21.4 ± 5.2	0.6635
Verbal fluency, Phonemic	43.8 ± 12.8	46.3 ± 11.3	0.4682
Stroop test	51.8 ± 7.1	52.8 ± 5.7	0.5777
Premorbid intelligence level			
WAIS IV, Vocabulary	14.1 ± 1.8	13.6 ± 2.1	0.4219

*Data are presented as mean ± SD. Statistics: Two-tailed t-test, *P < 0.05 compared to Middle-aged control group. Abbreviations: FCSRT, Free and Cued Selective Reminding Test; TMT, Trail Making Test; SDMT, Symbol Digit Modality Test; WAIS-IV, Wechsler Adult Intelligence Scale fourth edition; [1]results for FCSRT immediate recall were reported in De la Rosa et al. (2019).*

TABLE 3B | Cambridge Neuropsychological Test Automated Battery (CANTAB).

	Middle-aged controls *N* = 25	Middle-aged rugby players *N* = 24	*P*
Spatial working memory (SWM)			
Latency to first response	2,229 ± 1,245	2,215 ± 1,273	0.9620
Strategy	41.04 ± 6.28	42.00 ± 7.22	0.6185
Double Errors	0.08 ± 0.28	0.04 ± 0.21	0.9990
Paired association learning (PAL)			
First trial memory score	11.56 ± 3.70	12.65 ± 3.13	0.3001
Trials to success	2.34 ± 1.03	2.26 ± 0.92	0.8732
Stages completed	3.68 ± 0.56	3.78 ± 0.42	0.6467
Rapid visual information processing (RPV)			
Total hits	37.44 ± 7.95	37.30 ± 8.99	0.9877
Latency	449.2 ± 82.59	442.6 ± 62.15	0.8642
Correct Rejections	506.5 ± 20.14	507.7 ± 18.96	0.9959

Data are presented as median (IQR). Statistics: Mann–Whitney test.

(0.03,0.10), expressed as median (IQR).The Kruskal–Wallis test significance was $P = 0.0548$; Mann–Whitney test of paired data showed statistical differences between middle-aged controls and young controls ($P < 0.05$).

Neuropsychological Characterization

All middle-aged subjects demonstrated normality in their responses for the categories of memory, attention, psychomotor speed, executive functions, visual processing, and premorbid intelligence. Data are shown in **Table 3A** for standard tests and **Table 3B** for the computerized CANTAB battery. The rugby players showed significantly higher scores than the controls in the immediate memory recall of the FCSRT as previously reported (De la Rosa et al., 2019). No statistical differences were detected in any of the other parameters tested. However, the player group showed a trend ($P = 0.078$) of better executive function than the controls in the TMT Part B test. Neuropsychological results of both groups, controls and rugby players, were used here for statistical correlation study.

Statistical Correlations Between Categories of Parameters

The results of the analysis of correlations of gene expression with age, education, health and lifestyle characteristics for the three groups of the study; middle-aged controls, middle-aged rugby players and young controls, were used to build a heat map displayed in **Figure 3**. Significant Spearman's correlations showed values between /R/ = 0.41, $P < 0.05$ and /R/ = 0.74, $P < 0.001$. To highlight some of the findings, Spearman's analysis of sirtuin genes with diverse physical activity parameters showed a scattered positive correlation with SIRT2 and SIRT3 in players, negative correlations for SIRT1, SIRT2 and SIRT3 in middle-aged controls and negative correlations for SIRT2 in the young controls. In both control groups, IL10 levels positively correlated with fewer depression symptoms according to Hamilton-D. IL1B positively correlated with tobacco use and negatively correlated with vegetal servings in middle-aged controls. IL6 negatively correlated with the fruit servings in the diet of young controls. Antioxidant genes showed scattered

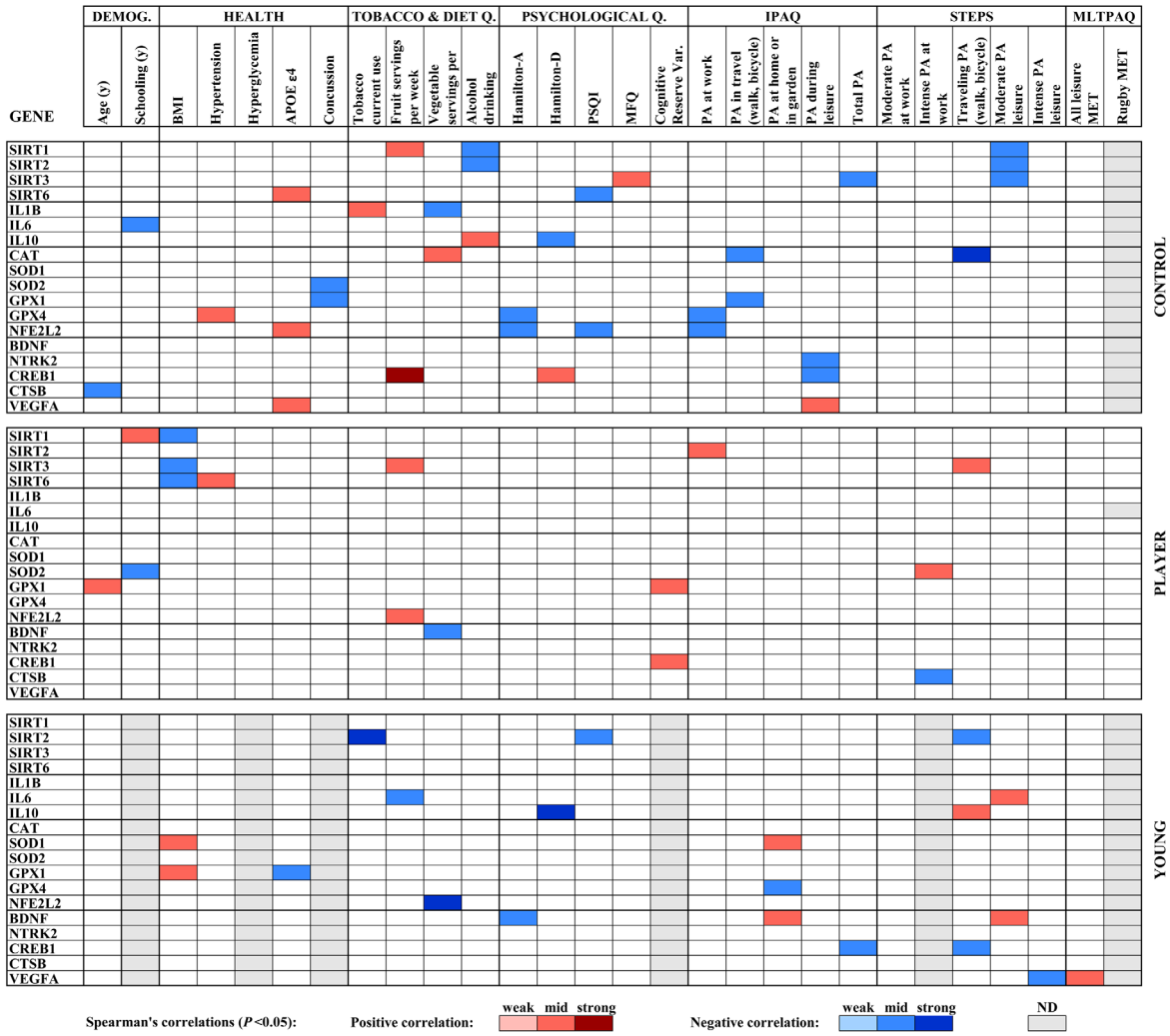

FIGURE 3 | Heat map built with Spearman's correlations between gene expression analysis and sociodemographic, health, diet and physical activity parameters obtained by standardized questionnaires. Potency of significant correlations ($P < 0.05$) were defined as weak (/R/ < 0.400), moderate (0.400 ≤ /R/ < 0.600) or strong (/R/ ≥ 0.600), as shown by color code.

negative correlations with physical activity parameters for CAT, GPX1, and GPX4 in middle-aged control group and positive correlations for SOD1 with home physical activity in young controls and for SOD2 with intense leisure physical activity in player group. BDNF was positively correlated with physical activity in the young control group. Other scattered correlations with physical activity included negative ones for CTSB in Players, positive ones for VEGFA in middle-aged controls and irregular VEGFA correlations in young controls. CREB1 negatively correlated with some parameters of physical activity in both control groups.

A heat map compiled with the significant correlations in gene expression with neuropsychological testing for both middle-aged groups, controls, and rugby players, as shown in **Figure 4**. Significant Spearman's correlations showed values ranged from /R/ = 0.41, $P < 0.05$, to /R/ = 0.58, $P < 0.01$. The player group showed positive correlations of SIRT1 for both cued subtests of FCSRT and WAIS-IV Inverse digits, as well as positive correlations for SIRT2 with both subtests of delayed FCSRT. However, the middle-aged control group showed scattered negative correlations for both genes and positive correlations between SIRT6 and PAL parameters. In the inflammatory status, IL6 expression negatively correlated with FCSRT and CANTAB PAL test parameters in the player group, whereas IL10 correlated with both cued subtests of FCSRT in the controls. Antioxidant enzyme genes in both groups also correlated positively with improved neuropsychological outcomes, such as the CAT gene with PAL parameters in players, SOD1 with SDMT and PAL parameters in controls, and SOD2 and GPX1 with verbal fluency also in controls. BDNF showed some generally negative correlations with neuropsychological scores in both groups, whereas CSTB and VEGFA showed a positive correlation with FCRT parameters in the player group.

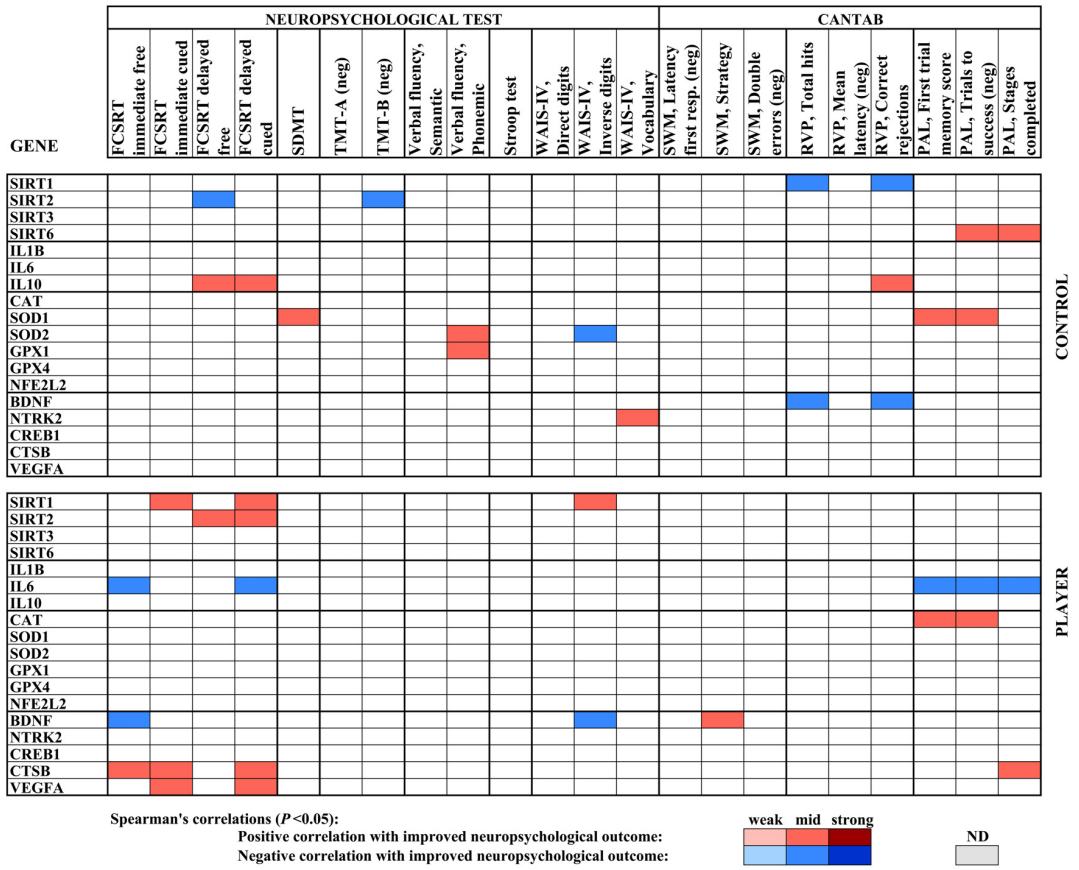

FIGURE 4 | Heat map built with Spearman's correlations between gene expression analysis and neuropsychological parameters of the middle age cohort. The sign of the correlation coefficient is reversed where the better neuropsychological outcome is measured by the lower value of the parameter, as indicated (neg). Potency of significant correlations ($P < 0.05$) were defined as weak ($/R/ < 0.400$), moderate ($0.400 \leq /R/ < 0.600$) or strong ($/R/ \geq 0.600$), as shown by color code.

The heat map built with the correlations between neuropsychological parameters and age, education, health and lifestyle characteristics for middle-age controls and rugby players is shown in **Figure 5**. Significant correlations showed values ranging from $/R/ = 0.41$, $P < 0.05$, to $/R/ = 0.70$, $P < 0.01$. Several parameters of higher physical activity positively correlated with a higher memory level in the FCSRT, which may underlie the significant improvement shown in the rugby player group. In the player group, yearly MET-h of rugby activity (MLTPAQ) correlated positively with both immediate and delayed free recall of FCSRT. MET-h of total leisure activity also correlated positively with the later measure. In the control group, STEPS and IPAQ parameters of leisure and domestic activity correlated positively with either cued or free delayed recalls. Correlation of physical activity parameters with other neuropsychological tests showed irregular outcomes. Namely, the control group showed a positive correlation between STEPS intense leisure physical activity and TMT-A performance. In the WAIS-IV, the Direct digits subtest correlated positively with IPAQ domestic physical activity in the controls, and with STEPS moderate physical activity at work in the player group. The WAIS-IV Vocabulary subtest positively correlated with IPAQ domestic physical activity in the player group. In the CANTAB battery, the SWM strategy showed positive correlations with IPAQ work and STEPS transportation in the player group and RPV latency decreased with MET-h of rugby activity in MLTPAQ. Furthermore, decreased RPV latency correlated with STEPS intense leisure activity in the controls. Among some scattered negative correlations, several IPAQ and STEPS physical activity parameters correlated with a poorer outcome in the RVP subtest of CANTAB in the player group. The health records for the player group showed a higher number of subjects with one or more episodes of a concussion than those in the control group. Concussions correlated negatively with performance in SDMT and TMT-A in the controls, TMT-A and B in the players, and RPV in both groups. Parameters of BMI, hypertension, and hyperglycemia showed irregular trends in the correlation with the neuropsychological test outcome. Presence of one APOE ε4 allele only correlated with lower semantic fluency and higher RPV latency in the player group. Higher depression scores in the Hamilton-D scale correlated negatively with FCSRT memory scores in three subtests in the control group. Poor sleeping habits according to the PSQI scale correlated with more CANTAB SWM errors in the controls. In

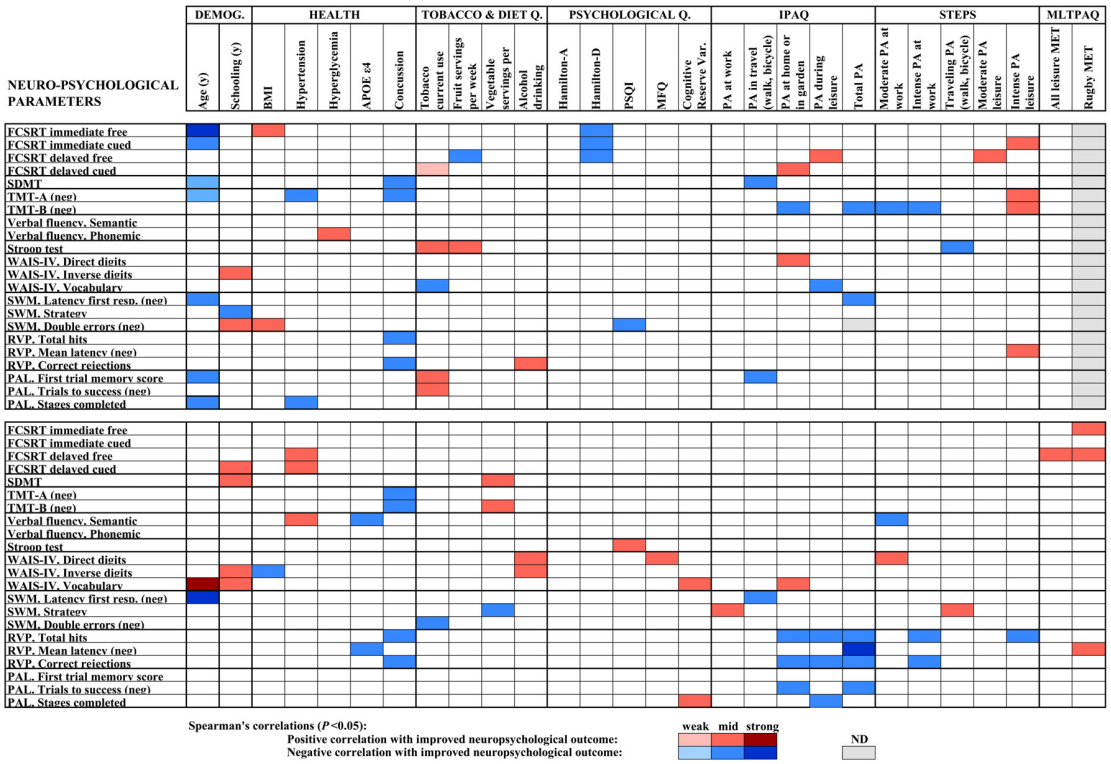

FIGURE 5 | Heat map built with Spearman's correlations between neuropsychological parameters of the middle-age cohort and sociodemographic, healthy diet and physical activity parameters obtained by standardized questionnaires. The sign of the correlation coefficient is reversed where the better neuropsychological outcome is measured by the lower value of the parameter, as indicated (neg). Potency of significant correlations ($P < 0.05$) was defined as weak ($/R/ < 0.400$), moderate ($0.400 \leq /R/ < 0.600$) or strong ($/R/ \geq 0.600$), as shown by color code.

diet habits, tobacco use showed an irregular outcome. Frequency of alcoholic drink consumption positively correlated with the score of Digit subtests of WAIS-IV in players and RPV correct rejections in controls. Increased vegetal servings correlated positively with better response in WAIS-IV Inverse digits but the worst strategy in SWM. As expected, increased years of schooling generally showed a positive correlation with better neuropsychological results; Specifically, with higher scores in the delayed cued memory subtest of FCSRT, SDMT and Vocabulary subtest of WAIS-IV in Players, with higher scores of WAIS-IV Inverse digits in both controls and players and with lower errors in the SWM subtest of CANTAB in controls. Lastly, age within the group negatively correlated with the neuropsychological performance in a number of parameters in middle-age controls but showed lower effect in rugby players.

DISCUSSION

Middle-aged amateur rugby players that had a long-term record of intense physical activity at leisure time, showed a distinct profile in whole blood gene expression compared to the age-matched controls with low sports activity. Furthermore, differences in the physical activity parameters showed some correlations with gene expression that supported the differential expression profile between rugby players and age-matched controls.

The central expression changes in the panel of 18 selected genes analyzed are those involving sirtuin genes SIRT1 and SIRT3. Rugby players maintained young-like levels of the sirtuin gene expression, whereas low-activity middle-aged controls showed a significant decrease. These findings in healthy middle-aged men may support the relevance of SIRT1 and SIRT3 as biomarkers of resilience against cognitive decline and/or in age-related frailty. Correlations of the expression of these genes with diverse physical activity parameters and neuropsychological parameters were generally positive in the player group and negative in the control subjects. The decreased expression of SIRT1 and SIRT3 genes in middle-aged controls compared to young controls is in agreement with previous reports on decreased serum levels of SIRT1 and SIRT3 proteins with aging (Kumar et al., 2014). These authors analyzed male subjects aged 60–80 years and proposed the lower circulating levels of SIRT1 and SIRT3 as markers of frailty. Much lower SIRT1 blood levels have been reported in cognitively impaired elders and AD patients (Kumar et al., 2013). In AD brains, there is a decrease in both the protein and mRNA of SIRT1 (Julien et al., 2009) and SIRT3 (Lee et al., 2018). Furthermore, decreases in SIRT3 have been associated with age-related hearing loss (Zeng et al., 2014) and age-exacerbated heart damage

(Porter et al., 2014). The group of rugby players maintained SIRT1 and SIRT3 expression at the level of the young subjects. The significantly increased physical activity of the player group compared to that of the age-matched controls may be the cause of their rejuvenation-like gene expression in blood cells. Similarly, male and female master athletes aged 60–70 years have shown higher mRNA and protein levels of SIRT1 and SIRT3 in skeletal muscle than aged-matched controls (Koltai et al., 2018). It is plausible that the levels of sirtuins in veteran sport practitioners, both track and field athletes, and rugby players, were also higher in the brain. Accordingly, experimental studies have demonstrated that chronic running exercise increases the protein levels of SIRT1 (Revilla et al., 2014) and SIRT3 (Bo et al., 2014) in the hippocampus of AD transgenic mice up to the levels of wild-type mice in conjunction with learning and memory normalization. The molecular mechanisms underlying the neuroprotective functions of SIRT1 have been studied in animal models. This nuclear-located sirtuin senses nutrient or stress signaling and responds activating pathways of synaptic plasticity and cognition (Gao et al., 2010; Michán et al., 2010). SIRT1 overexpression in the hippocampus of either AD transgenic mice or wild type mice induces neurotrophic factors, improves proteostasis of abnormal proteins and induces cognitive enhancement (Corpas et al., 2017). The later results that show improvement of neuroprotective mechanisms in normal healthy mice indicate the potential for brain resilience of SIRT1. Indeed, here we found that SIRT1 gene expression in whole blood positively correlated with parameters of memory and executive functions in players, but not in middle-aged controls. Furthermore, SIRT1 activates anti-inflammatory and antioxidant pathways, improves insulin sensitivity and other metabolic aspects, and delays senescence in brain and peripheral organs (Haigis and Sinclair, 2010). SIRT3 controls many functional aspects of mitochondria, the energy generator of the cell, and therefore has a significant role in healthy aging (McDonnell et al., 2015). SIRT3 is involved in cell metabolism and DNA repair in a variety of tissues, and also activates antioxidant genes such as SOD2 and CAT (Ansari et al., 2017). Its neuroprotective mechanisms have been less studied than those of SIRT1. However, SIRT3 has shown to mediate adaptive response of neurons to metabolic and oxidative stress (Cheng et al., 2016; Li et al., 2018). Therefore, similarly to SIRT1, SIRT3 activation would increase resilience against neurodegeneration. Not unexpectedly, there is a close relationship between both SIRT1 and SIRT3. SIRT3 is a substrate for SIRT1 deacetylation and therefore its activation level may be directly modulated by SIRT1 (Kwon et al., 2017). In addition, they deacetylate some homologous substrates present in their corresponding cell compartments, nucleus and mitochondria (Hirschey et al., 2011), and may act cooperatively in response to inducing factors (Bell and Guarente, 2011) or against pathological conditions (Kwon et al., 2017; Chen et al., 2018). Therefore, we propose that the maintenance of the SIRT1-SIRT3 axis functionality, both in the brain and peripheral tissues, is a molecular mechanism that contributes to the acquisition of resilience through long-term physical activity. SIRT2 and SIRT6 did not show differential expression based on physical activity or age of the groups. The role of SIRT2 in the regulation of aging is not clarified. SIRT2 protein levels decrease in blood serum of frail patients, but less significantly than SIRT1 and SIRT3 (Kumar et al., 2014), and it was suggested as being a marker of senescence and neurodegeneration (Theendakara et al., 2013). However, it also may promote longevity (North et al., 2014). SIRT6 is an anti-aging sirtuin that regulates metabolism and genome stability (Kuang et al., 2018). Its functions of DNA repair and cell survival are particularly neuroprotective in the brain, where it decreases with aging and AD (Kaluski et al., 2017). Absence of any change in the expression of SIRT2 and SIRT6 gene may, therefore, be indicative of the health status of all the subjects analyzed.

Antioxidant genes CAT and SOD1 showed a striking similarity to SIRT1 and SIRT3 in their response to physical activity. Players showed a young-like level of CAT and SOD1 expression and middle-aged controls a significant decrease of them. They might be activated downstream of sirtuin genes in response to physical exercise. Activities of CAT and SOD enzymes are known to increase in rodent brain submitted to physical training (de Souza et al., 2019). Catalase is a first-line antioxidant that decomposes hydrogen peroxide. It may have an anti-aging role as seen by increased CAT gene expression in the liver of long-lived mouse models and decreased CAT expression in short-lived models (Brown-Borg and Rakoczy, 2000). Here we demonstrated a decrease in gene expression in whole blood with age. Increased CAT expression might be neuroprotective given that, for instance, CAT expression correlated positively with visual memory in the player group. The cytosolic enzyme Cu/ZnSOD is the main responsible for the decomposition of superoxide radicals. It has been reported a decrease of Cu/ZnSOD levels in the brain of aged AD transgenic female mice that were normalized by a neuroprotective therapy of physical exercise (García-Mesa et al., 2016). We demonstrated gene expression changes in blood cells but we did not detect changes of SOD enzymatic activity, indicating that oxidative stress processes at middle age are mild. Indeed, our previous study showed no differences in plasma oxidized proteins or lipids between middle-aged controls and young controls (De la Rosa et al., 2019). The genes SOD2 and GPX1 that code for the antioxidant enzymes MnSOD and GPx1 did not show differences in expression levels with age or physical activity in whole blood. MnSOD decomposes superoxide anion in the mitochondria. GPx1 is the more abundant GPx isoenzyme and reduces mainly hydroperoxides in the cytoplasm. Both enzymes have shown altered brain activity levels in animal models of AD with elevated oxidative stress (García-Mesa et al., 2016). Therefore, lack of changes in these widely expressed antioxidant genes would also support the absence of oxidative processes of neurodegeneration or other chronic diseases in apparently healthy middle-aged subjects. However, we found a decrease in the expression of the gene GPX4 coding for the GPx4 isoenzyme in both middle-aged controls and players. Enzymatic levels of this isoform are much lower than those of GPx1, although it shows high affinity for lipid peroxides and has been suggested as being neuroprotective (Cardoso et al., 2017). Further studies are needed to understand the relevance of GPX4 expression changes in aging

in diverse tissues. The expression profile of NFE2L2 was also lower in both middle-aged groups. There is no consensus on the age-related changes of the protein levels of Nrf2 and its signaling activity but the decline of this pathway induces premature aging (Kubben et al., 2016).

The cytokine IL1B is an important mediator of the inflammatory response. Its decreased level in the middle-aged rugby players as compared to young controls confirmed the reported anti-inflammatory effect of physical activity at older ages (Jankord and Jemiolo, 2004). Although no differences were found between middle-aged controls and young controls in the gene expression, protein levels of IL1B in plasma showed a trend to increase in middle-aged controls as compared to young control. Therefore, the changes of age-related inflammation are probably not yet clearly detectable at middle age. No differences were found in the levels of gene expression of IL6 and IL10 among the groups. Gene expression of pro-inflammatory or anti-inflammatory markers may be lower in the blood than in other compartments (Pilling et al., 2015). However, it is interesting to note that the expression level of pro-inflammatory cytokine IL6 negatively correlated with memory and visual processing parameters in the rugby players and that the level of expression of anti-inflammatory cytokine IL10 positively correlated with scores for memory and attention and executive function in controls. Anti-inflammatory changes may be downstream of sirtuin activation through NF-κB modulation.

Expression levels of analyzed neurotrophic factors and related genes in whole blood did not increase in the middle-aged rugby players as compared to middle-aged control that would support a further contribution to the proposed anti-aging effects of physical exercise. The expression of the trophic factor genes BDNF and VEGFA and the myokine gene CSTB showed a tendency to decrease at middle age compared to young age. Such behavior was significant in the players' group for BDNF and VEGFA. No changes were detected for the BDNF receptor gene TRK. The expression of CREB1 also tended to decrease with age, with such a change being most significant for the control group. CREB1 signaling is activated after endurance exercise to induced downstream protective genes (Neubauer et al., 2014). CREB1 is a transcriptional activator involved in sirtuin signaling as well as in the regulation of neurotrophic factors such as BDNF. BDNF is synthesized in the brain but also in muscle and other peripheral compartments. BDNF has been suggested as being a key mediator for increases in the volume of the hippocampus and related cognitive benefits of physical exercise (Erickson et al., 2011). The role of the cysteine protease CSTB in the brain is poorly understood, but its release from skeletal muscle following exercise could induce cognitive benefits through several pathways including induction of BDNF and neurogenesis (Moon et al., 2016). VEGFA is released mainly from the endothelial cells of muscle capillaries following acute exercise and it acts synergistically with the cascade of trophic factors (Archer, 2011). These analyses were performed under resting conditions, where we had previously reported a decrease in serum levels of BDNF and CTSB proteins in young and middle-aged trained men as compared to age-matched sedentary subjects (De la Rosa et al., 2019). Therefore, we cannot discard that the transduction of some trophic factors sensitive to the exercise may decrease during the resting period as found here for BDNF and VEGF. However, VEGFA and CTSB expression correlated positively with declarative memory parameters in the player group, although not BDNF, TRK or CREB1. We could not determine the gene expression of another two trophic factors induced by physical activity; IGF1 which is deeply involved in the neurogenesis mediated by exercise (Trejo et al., 2001) and the neuroprotective GDNF (Revilla et al., 2014), because they had low expression in the whole blood samples.

The absence of differences in sociodemographic variables, and health and lifestyle parameters other than leisure physical activity between both middle-aged groups indicates that long-term engagement in playing amateur rugby may underlie the differential gene expression. As discussed above, the changes found in the sirtuin genes in blood cells are in agreement with the results in brain tissue after physical exercise reported in previous studies in experimental models of aging and neurodegeneration. Furthermore, sirtuins have experimentally proved pro-cognitive effects. Here, the expression levels of genes SIRT1, SIRT3, and downstream CAT and SOD1 in the player group were similar to those in the young controls, but middle-aged controls showed a significant decrease compared to young age levels. These results paralleled a better memory outcome in rugby players that in middle-aged controls. We speculate that the maintenance of the peripheral axis SIRT1-SIRT3 in middle-aged active men reflects the brain status and it may contribute to the prevention of age-associated cognitive loss and neurodegeneration through physical exercise.

It is likely that the findings in this study can be extended to middle-aged women. We have not been able to enroll rugby player women, but previous studies have reported benefices of physical activity in the cognitive health of aged women (Yaffe et al., 2001; Lin et al., 2019). Furthermore, as discussed above, old female master athletes have shown higher mRNA and protein levels of SIRT1 and SIRT3 in skeletal muscle than aged-matched controls (Koltai et al., 2018) suggesting activation of SIRT1-SIRT3 axis. Therefore, no differential gender response is anticipated.

Not less important, a leisure sport activity practiced in a team such as amateur rugby might facilitate to comply with the volume of physical activity required to maintain health and wellbeing. In most medium and high-income countries, there is an urgent call to implement programs, practices, and policies of physical exercise at all ages (Piercy et al., 2018). Resilience findings at middle age in amateur rugby players can help clinicians monitor and improve the cognitive health of the aging population.

LIMITATIONS OF THE STUDY

Incidences of concussion in the player group were higher than in the controls and we cannot exclude some effect of this factor in the parameters analyzed. Nevertheless, negative correlations between some IPAQ and STEPS parameters and CANTAB RPV and PAL performance in the player group may suggest the presence of specific minor impairments in the subjects affected by one or more episodes of mild brain

trauma. However, these mild accidents did not cause worsening of the neuropsychological response, as in contrast, rugby players showed some memory improvements compared to age-matched controls. Although rugby is a contact sport, the risk of moderate or severe traumatic brain injury is low. Rugby playing is generally less harsh than American football and no effects of the later have been reported for all-cause mortality in retired football players (Venkataramani et al., 2018).

The volume of physical exercise of each participant in the study was obtained from standardized questionnaires. In the absence of physiological measures of fitness, we cannot discard some inaccuracies in the time or the intensity of the exercise performed.

The cross-sectional design of the study introduces some weakness because it may preclude the detection of possible bias in the groups. However, the long-term sports activity clearly differentiates the rugby player group from the control group. Middle-aged controls had lower leisure physical activity but otherwise had similar behaviors and educational level. Furthermore, in the players' group, whole year METs-h spent in rugby playing correlated with higher declarative memory in FCSRT; thus, supporting the positive effect of the sport on this collective of long-time practitioners of amateur rugby.

CONCLUSIONS

This study supports the beneficial effect of long-term practice of leisure sport as an anti-aging and neuroprotective lifestyle and provides further evidence of SIRT1 and SIRT3 gene activation by physical activity.

The young-like levels of SIRT1, SIRT3, CAT, and SOD1 gene expression and the lower expression of IL1B, determined in peripheral blood of middle-aged amateur rugby players, suggest that the SIRT1-SIRT3 axis and downstream genes may contribute to the beneficial effects of physical activity. Similar changes in the brain may be a factor of anti-age resilience.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Hospital Clinic de Barcelona, Spain (Reg. HCB/2014/0759). Written informed consent was obtained from all participants and all procedures were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

AUTHOR CONTRIBUTIONS

JV, ER-F, MP, DB-F, MG-C, and CS contributed to the conception and design of the study. DB-F, MG-C, and CS obtained ethical approval and jointly supervised the study. ES conducted the lifestyle questioning and neuropsychological testing. RC, AR, and MO obtained and processed blood samples. RC, AR, SS, CG-F, EC, and CS did the experimental analysis and data processing. CS wrote the manuscript draft. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Sedentary Behavior and Problematic Smartphone Use in Chinese Adolescents: The Moderating Role of Self-Control

*Ming-Qiang Xiang[1†], Long Lin[1†], Zi-Rong Wang[2], Jin Li[2], Zebo Xu[1,3] and Min Hu[1]**

[1] Department of Sports and Health, Guangzhou Sport University, Guangzhou, China, [2] Department of Graduation, Guangzhou Sport University, Guangzhou, China, [3] Department of Linguistics and Modern Languages, The Chinese University of Hong Kong, Sha Tin, China

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Henriette van Praag,
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University of Murcia, Spain

***Correspondence:**
Min Hu
whoomin@aliyun.com

[†]These authors have contributed equally to this work and share first authorship

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This study investigated smartphone use characteristics including the purpose of smartphone use (i.e., leisure, learning, or work) and situational smartphone use (i.e., sitting, standing, or moving about) in Chinese adolescents. Moreover, it tested the moderating role of self-control in the link between sedentary behavior and problematic smartphone use. A total of 947 adolescents completed measures of the purpose of their smartphone use, situational smartphone use, sedentary behavior, self-control, time on smartphone, and smartphone addiction. Results showed that the majority of smartphone use was for leisure and learning, and 90.9% of adolescents reported typically sitting as they used the smartphone. Problematic smartphone use was positively correlated with sedentary behavior and negatively correlated with self-control. Moreover, the relationship between sedentary behavior and problematic smartphone use was moderated by self-control, in that the negative correlation was stronger for adolescents with low self-control and weaker for those with high self-control. These results contribute to the understanding of when sedentary behavior is associated with problematic smartphone use. Several limitations and implications are discussed in this study.

Keywords: sedentary behavior, problematic smartphone use, self-control, adolescents, exercise interventions

INTRODUCTION

With the development of internet-based smart devices, the prevalence of smartphone use has steadily increased worldwide, including in China. The Ministry of Industry and Information Technology of China (2019) announced that more than 1.57 billion Chinese people had their own mobile phones in 2018. Although the smartphone brings conveniences to people's digital lifestyle, many problematic smartphone usages have also emerged among younger people, including in Chinese adolescents (Liu et al., 2017, 2018a). With its special features of convenience, immediacy, and affordability, today's smartphone allows people to call, receive and send messages, surf the internet, play mobile games, and update social networking sites (e.g., Facebook and WeChat) almost anywhere and anytime. Historically, these

activities were defined as sedentary behaviors (Rosenberg et al., 2010). More importantly, a consistent body of literature showed that more than 80% of people reported typically sitting when using their device (Barkley and Lepp, 2016; Long et al., 2016; Lee et al., 2017; Fennell et al., 2019), and such inactive behaviors are linked to numerous comorbidities including obesity, cardiovascular disease, and metabolic syndrome (Owen et al., 2010). Due to the prevalence of smartphone usage and the access it provides to sedentary behaviors, it is important to expand our understanding of the behavioral health implications potentially related to smartphone use. This study considers the relationship between sedentary behavior and problematic smartphone use and tests the moderating role of self-control in the relationship between those variables.

Sedentary Behavior and Problematic Smartphone Use

An excessive amount of sedentary behavior in adolescents is a growing problem in China (Lu et al., 2017). Although many factors may affect sedentary behavior, the association of smartphone use with sedentary behavior and leisure time is well demonstrated, much as sedentary behavior has been linked with traditional forms of screen-based activities (e.g., watching television, playing video games, and surfing the internet). For example, 70% of college students and 81% of adults reported using their smartphone for leisure purposes (Barkley and Lepp, 2016; Fennell et al., 2019). Prior research has also found that sedentary behaviors are strong predictors of smartphone usage time in college students as well as adults aged 18–80, indicating that excessive smartphone use may increase sedentary behaviors and distract from physical activity (Barkley and Lepp, 2016; Fennell et al., 2019). Furthermore, after controlling for other factors linked to physical quality (e.g., gender, percentage of body fat, and self-efficacy for exercise), excessive smartphone use can ultimately result in reduced cardiorespiratory fitness levels among college students (Lepp et al., 2013).

Prior studies have focused mainly on college students or adults. However, little is known about the relationship between sedentary behavior and problematic smartphone use in adolescents. Adolescence represents a critical transitional stage of development, during which personal lifestyle choices and behavior patterns are established. Clearly, more studies are required to explore smartphone use characteristics as well as the relationship between sedentary behavior and problematic smartphone use in adolescents.

Self-Control as a Moderator

Self-control, defined as the ability to volitionally control or override inner desires and external temptations in order to achieve long-term goals (Tangney et al., 2004), is an important dispositional trait for generating adaptive personal and social responses. High self-control is positively associated with desirable life outcomes, including better physical and mental health, higher academic performance, and more wealth (Tangney et al., 2004; Moffitt et al., 2011). In contrast, a deficit in self-control is positively associated with undesirable outcomes or social problems, such as binge eating, aggression, depression, and addiction (Denson et al., 2011; Özdemir et al., 2014; Pearson et al., 2018). Problematic smartphone use is generally described as an addictive behavior or incapacity to control cravings to use smartphones (Walsh et al., 2010; Liu et al., 2018b). According to self-regulation theory, addictive behaviors primarily result from failures of self-regulation. Poor self-control might limit an individual's ability to reduce cravings and restrain addiction (Köpetz et al., 2013; Gökçearslan et al., 2016). This lack of self-control is intrinsically linked to problematic smartphone use. Indeed, a consistent body of research has shown that low levels of self-control not only predict high-frequency usage of smartphones (Wilmer and Chein, 2016; Berger et al., 2018) but also link to smartphone addiction such as withdrawal symptoms, mood changes and cyberspace-oriented relationship (Gökçearslan et al., 2016; Jiang and Zhao, 2016; Yun et al., 2016; Berger et al., 2018).

Self-control is also correlated with sedentary behavior. For example, preliminary evidence showed that lower inhibition-control was directly or indirectly associated with sedentary behavior (Hoang et al., 2013). In modern life, although individuals often plan and intend to exercise, they do not always transform their intentions into actual exercise behavior. According to behavioral economics theory, sedentary behavior can be perceived as an easy, "low-cost" activity with immediate reinforcements, such as fun and entertainment, whereas physical activity can be viewed as a "high-cost" commitment, requiring effort and few immediate reinforcements (Epstein, 1998). Thus, Martin Ginis and Bray (2010) suggested that the capacity to block out sedentary behavior and promote physical activity requires self-control.

With in-depth study, self-control not only negatively correlated with personal and social problems, but also played an important, protective moderator role in the relationship between negative factors and their outcomes. Cooper et al. (2017) found that self-control could buffer the correlation between school burnout and emotional dysregulation. Furthermore, Liu et al. (2018b) found that the direct association between mindfulness and poor sleep quality and the indirect association through rumination were both moderated by self-control among adolescents. These two associations are stronger for those with low self-control and weaker for those with high self-control.

To the best of our knowledge, it remains unclear how sedentary behavior and self-control interact to affect problematic smartphone use. To fill these gaps, it is worth constructing a moderation model to test the moderating variable of self-control in the association between sedentary behavior and problematic smartphone use. The moderation model would contribute to understanding of how self-control protects individuals from problematic smartphone use.

Hypotheses

This study aims to investigate smartphone use characteristics and explore the relationship between sedentary behavior, self-control and problematic smartphone use in Chinese adolescents. Specifically, we hypothesized the following in a sample of adolescents.

Hypothesis 1: Because a smartphone provides a variety of leisure (e.g., videos and game) and learning (e.g., English materials) applications, the majority of smartphone use will be for leisure and learning purposes in Chinese adolescents.

Hypothesis 2: Because the smartphone makes it easier to access traditionally sedentary and screen-based activities, smartphone use will occur primarily while sitting.

Hypothesis 3: Because smartphone use primarily occurs while sitting, the sedentary behavior will be positively related to problematic smartphone behaviors.

Hypothesis 4: Because prior researches have indicated that self-control plays an important protective role, the relationship between sedentary behaviors and problematic smartphone use was moderated by self-control.

MATERIALS AND METHODS

Participants and Data Collection

We used a descriptive transversal design study which was approved by the Human Experimental Ethics Board of Author's University (Reference number: 2018LCLL-007). With a convenient sampling method, we recruited students from two junior high schools (grade 7 to grade 9) and two senior high schools (grade 10 to grade 12) in the Guangdong province in southern China. In each target school, we randomly chose two or three classes in each grade. Prior to investigation, the parents or guardians of participants were well-informed and their written consent was obtained. A total of 969 Chinese target students were invited to voluntarily participate in the anonymous paper-and-pencil questionnaires survey, which was conducted in classrooms by well-trained college students. All participants completed our survey, but 22 participants were excluded because of missing data on the main variables. Overall, 947 adolescents in the sample were employed, the mean age was 14.13 (SD = 1.79) ranging from 11 to 18 years. There were 489 male students with an average age of 14.13 (SD = 1.71) and 458 female students with an average age of 14.12 (SD = 1.89).

Measurements

Smartphone Use Characteristics

The study evaluated basic demographics (e.g., age, gender, grade, and smartphone ownership), purpose of smartphone use, and situational smartphone use. Regarding purpose of smartphone use, participants were asked to indicate "what percentage of the time the smartphone is used for the following purposes: leisure, learning, work." The list of items was designed to ensure that the sum of the three responses totaled 100% (Lepp et al., 2014). The situational smartphone use was assessed with three fixed choice items: "When I am using my smartphone, I am most often: (a) sitting, (b) standing, or (c) moving about."

Sedentary Behavior

Sedentary behavior (i.e., sitting) was assessed with two items from the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003; Bauman et al., 2009). Participants reported the average number of minutes of each week day (or each weekend day) they spent sitting. Weekly sedentary behavior was calculated using the following equation: weekly sedentary behavior = [(5 × minutes of sitting per week day) + (2 × minutes of sitting per weekend day)]/7.

Self-Control

We used the China short form of the trait self-control scale (SCS) (Tan, 2008) revised from the original version by Tangney et al. (2004), including a scale of 19 items. The SCS measures five aspects of self-control abilities:(1) deliberate and non-impulsive action, (2) healthy habits, (3) resistance to temptation, (4) work ethic, and (5) moderation in seeking diversions. Participants assessed each item on a five-point scale from 1 (not at all like me) to 5 (very much like me). Higher scores on this scale indicate a stronger capability for self-control.

Problematic Smartphone Use

Two questionnaires were selected to assess the problematic smartphone use, including time on smartphone use and smartphone addiction scale.

Time on smartphone was assessed with two items using a method followed by Lepp et al. (2014). Participants were asked to estimate their average time spent (in minutes) on their smartphone for each weekday and each weekend day. This self-report measure is associated with objectivity and other self-reported measures of smartphone use, which were applied in previous studies (Barkley and Lepp, 2016; Fennell et al., 2019). Weekly smartphone use was calculated using the following equation: weekly smartphone use = [(5 × minutes of smartphone use per week day) + (2 × minutes of smartphone use per weekend day)]/7.

Smartphone addiction was assessed by the ten-item Smartphone Addiction Scale-Short Version (SAC-SV) for adolescents (Kwon et al., 2013). The scale was translated by independent researchers using the parallel translation method. Any disagreement was resolved by discussion or, if required, by consulting a third author. Participants assessed each item on a six-point scale: 1 (fully disagree) to 6 (fully agree). The total scores ranged from 10 to 60. Kwon et al. (2013) suggested cut-off points per gender (boys 31 and girls 33) to classify the smartphone addiction group (SAG) or non-smartphone addiction group (non-SAG).

Statistical Analysis

All data analysis was performed using SPSS 23.0. A p-value of 0.05 indicated statistical significance. We first computed descriptive statistics for the whole sample, and then compared differences between SAG and non-SAG with continuous variables using independent t-test and categorical variables using χ^2. Additionally, we used a Pearson correlation analysis to assess the association between sedentary behavior, physical activity, self-control, smartphone use, and smartphone addiction. Finally, we performed moderation analyses using Hayes (2013) bootstrapping Process for SPSS (Model 1) to examine whether the sedentary behavior effect on time of smartphone use and smartphone addiction were moderated by self-control. All

TABLE 1 | Comparisons of variables between subjects with and without SA.

	All (n = 947)	Non-SAG (n = 776)	SAG (n = 171)
Age (years)	14.13 ± 1.79	14.05 ± 1.84	14.47 ± 1.55**
Gender			
Boys	489 (51.6%)	400 (81.8%)	89 (18.2%)
Girls	458 (48.4%)	376 (82.1%)	82 (17.9)
Ratio of smartphone ownership	698 (73.7%)	557 (71.8%)	141 (82.5%)**
Purpose of smartphone use (%)			
Leisure	43.87 ± 25.65	40.96 ± 24.48	57.06 ± 26.78***
Learning	45.94 ± 24.81	48.85 ± 24.20	32.75 ± 23.28***
Work	9.59 ± 13.56	9.54 ± 13.41	9.82 ± 14.27
Situational of smartphone use			
Sitting	861 (90.9%)	711 (91.6%)	150 (87.7%)
Standing	24 (2.5%)	20 (2.6%)	4 (2.3%)
Moving about	62 (6.5%)	45 (5.8%)	17 (9.9%)
Sedentary behavior (min/day)			
Weekday	465.99 ± 126.89	461.52 ± 125.12	485.76 ± 133.01*
Weekend	382.32 ± 143.82	371.57 ± 140.08	424.67 ± 149.82***
Total	442.08 ± 112.74	436.31 ± 110.40	468.30 ± 119.68**
Self-control	3.59 ± 0.53	3.68 ± 0.51	3.21 ± 0.43***
Smartphone use (min/day)			
Weekday	13.95 ± 33.15	12.58 ± 30.47	20.15 ± 42.84*
Weekend	171.84 ± 158.16	150.85 ± 141.52	267.09 ± 191.69***
Total	59.06 ± 54.61	52.09 ± 48.47	90.70 ± 68.25***
Smartphone addiction	25.09 ± 7.44	22.59 ± 5.35	36.46 ± 4.39***

*SAG, smartphone addiction group; Non-SAG, non-smartphone addiction group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

continuous variables were standardized and the interaction terms were computed based on standardized scores. The bootstrapping method produced 95% bias-corrected confidence intervals of these effects from 5000 resamples of the data (Hayes, 2013).

RESULTS

Descriptive Statistics and Comparative Analysis

Based on of results of previous studies (Kwon et al., 2013; Lopez-Fernandez, 2017), the scoring 32 was selected as the cut-off to identify smartphone addiction because there was no significant difference between gender in SAS-SV scores (t = 0.69, p = 0.49). **Table 1** presented the socio-demographic and smartphone use characteristics between those with and without SA.

A total of 947 adolescent subjects participated in this study; 698 of the participants (73.7%) owned a smartphone with 776 of them in the non-SAG (81.9%) and 171 in the SAG (18.1%). When these two groups were compared, there were no significant differences in their genders; however, the age was significantly greater in the SAG (t = 2.76, p = 0.006), and the proportion of smartphone ownership was also significantly higher in the SAG (χ^2 = 8.24; p = 0.004).

Regarding purpose of smartphone use, on average, participants categorized 43.87% of their smartphone use as leisure, 45.94% as learning, and 9.59% as work. On close inspection, the SAG had significantly greater smartphone use as leisure (t = 7.92, $p < 0.001$), but lower smartphone use as learning (t = -7.65, $p < 0.001$) compared with the non-SAG. In this sample, 90.9, 2.5, and 6.5% of participants reported that they are most likely sitting down, standing and moving, respectively, while using their smartphone. However, there was no significant difference in usage preferences between SAG and non-SAG regarding these three postures (χ^2 = 0.014, p = 0.906).

The mean daily sitting was 442.08 (SD = 112.74) min/day. The mean self-control score was 3.59 (SD = 0.53) units. When these two groups were compared, the SAG showed significantly higher sedentary behavior on weekdays (t = 2.26, p = 0.024) and weekends (t = 4.43, $p < 0.001$), as well as higher overall minutes being sedentary (t = 3.38, $p < 0.001$). They also showed significantly lower self-control than the non-SAG (t = 11.17, $p < 0.001$).

Regarding daily smartphone use, mean smartphone use time in weekdays, weekends, and total minutes was 13.95 (SD = 33.15), 171.84 (SD = 158.16), and 59.06 (SD = 54.61) min/day, respectively. The SAG had significantly greater smartphone use

TABLE 2 | Descriptive statistics and correlations between variables.

Variables	M	SD	1	2	3
1. Sedentary behavior	442.08	112.74			
2. Self-control	3.59	0.53	−0.07*		
3. Time on smartphone use	59.06	54.61	0.23***	−0.23***	
4. Smartphone addiction	25.09	7.44	0.12***	−0.54***	0.34***

*N = 947. *$p < 0.05$, ***$p < 0.001$.*

TABLE 3 | Moderation analysis.

Outcomes	Predictors	β	*t*	LLCI	ULCI
Time on smartphone use	Gender	−0.07	−1.22	−0.19	0.04
	Age	0.24	7.50***	0.17	0.30
	Sedentary behavior	0.15	4.85***	0.09	0.21
	Self-control	−0.18	−6.04***	−0.24	−0.12
	Sedentary behavior × self-control	−0.08	−2.66**	−0.13	−0.02
Smartphone addiction	Gender	0.01	0.18	−0.10	0.12
	Age	0.04	1.45	−0.01	0.10
	Sedentary behavior	0.06	2.17*	0.01	0.12
	Self-control	−0.53	−19.33***	−0.59	−0.48
	Sedentary behavior × self-control	−0.06	−2.23*	−0.11	−0.01

*n = 947. Bootstrap sample size = 5000. CI, confidence interval; LL, low limit; UL, upper limit. *p < 0.05, **p < 0.01, ***p < 0.001.*

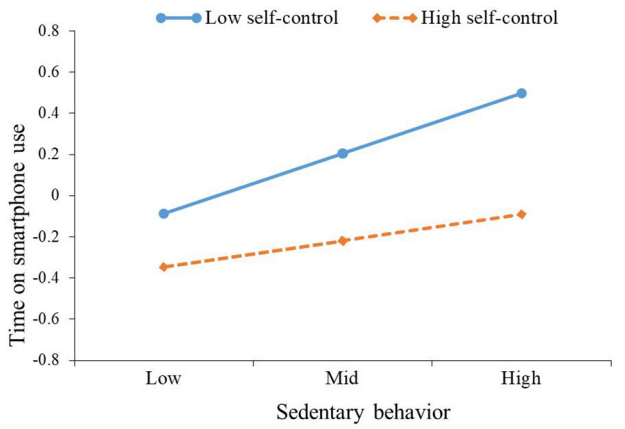

FIGURE 1 | The plot of the relationship between sedentary behavior and time on smartphone use at two levels of self-control.

weekdays (t = 2.71; p = 0.007), weekends (t = 9.07; $p < 0.001$), and for total minutes of use (t = 8.69; p = 0.005).

Correlation Analyses

The descriptive statistics and correlation matrix are presented in **Table 2**. Sedentary behavior was positively correlated with time on smartphone use ($p < 0.001$) and smartphone addiction ($p < 0.001$), and negatively correlated with self-control ($p < 0.05$). Self-control was negatively associated with time on smartphone use ($p < 0.001$) and smartphone addiction ($p < 0.001$).

Testing for the Moderation Model

The main results of moderation analysis generated by Hayes (2013) SPSS macro PROCESS are presented in **Table 3**. Regarding time on smartphone use, after controlling for gender and age, sedentary behavior was positively correlated with time on smartphone use (β = 0.15, $p < 0.001$); self-control was negatively correlated with time on smartphone use (β = −0.18, $p < 0.001$); and the interaction of sedentary behavior and self-control was negatively correlated with time on smartphone use (β = −0.08, $p < 0.01$). Namely, self-control moderated the association between sedentary behavior and time on smartphone use. To better understand the moderating effect of self-control, the plot of the relation between sedentary behavior and time on smartphone use at two levels of self-control (1 SD below the mean and 1 SD above the mean) was described in **Figure 1**. As can be seen from **Figure 1** and the conditional effects analysis in **Table 4**, for individuals with low self-control (1 SD below the mean), sedentary behavior was positively associated with time on smartphone use (β = 0.23, $p < 0.001$), while this association (β = 0.07, $p > 0.05$) was not significant for individuals with high self-control (1 SD above the mean).

As can be seen from the moderation model for predicting smartphone addiction, after controlling for gender and age, sedentary behavior was positively correlated with smartphone addiction (β = 0.06, $p < 0.05$), while the interaction of sedentary behavior and self-control was negatively correlated with smartphone addiction (β = −0.06, $p < 0.05$). In other words, self-control moderated the association between sedentary behavior and smartphone addiction. The plot of the relation between sedentary behavior and smartphone addiction at two levels of self-control (1 SD below the mean and 1 SD above the mean) was described in **Figure 2**. As can be seen from **Figure 2** and the conditional effects analysis in **Table 4**, for individuals with low self-control (1 SD below the mean), sedentary behavior was positively associated with smartphone addiction (β = 0.12, $p < 0.01$), while this association (β = 0.01, $p > 0.05$) was not significant for individuals with high self-control (1 SD above the mean).

DISCUSSION

A descriptive transversal design was carried out to investigate smartphone use characteristics in this study, including the purpose of smartphone use (i.e., leisure, learning, or work) and situational smartphone use (i.e., sitting, standing, or moving about). Furthermore, this study examined the moderating role of self-control between sedentary behavior and problematic smartphone use in Chinese adolescents. In the current study, the proportion of adolescents who own a smartphone is 73.7% among Chinese adolescents. They spend more time on their

TABLE 4 | Conditional effects results at values of moderators.

Outcomes	Predictor	Values of self-control	β	*t*	LLCI	ULCI
Time on smartphone use	Sedentary behavior	Mean - SD	0.23	5.47***	0.15	0.31
		Mean	0.15	4.85***	0.09	0.21
		Mean + SD	0.07	1.69	−0.01	0.16
Smartphone addiction	Sedentary behavior	Mean - SD	0.12	3.17**	0.05	0.19
		Mean	0.06	2.17*	0.01	0.12
		Mean + SD	0.01	0.06	−0.08	0.08

*n = 947. Bootstrap sample size = 5000. CI, confidence interval; LL, low limit; UL, upper limit. *p < 0.05, **p < 0.01, ***p < 0.001.*

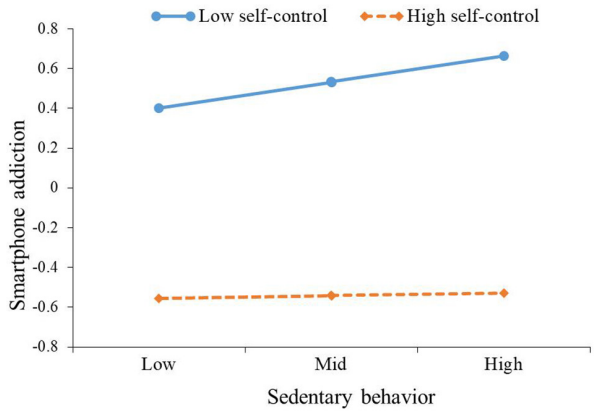

FIGURE 2 | The plot of the relationship between sedentary behavior and smartphone addiction at two levels of self-control.

smartphone on weekends because of the heavy learning tasks and explicit prohibition in school on smartphone use at weekday (Gao et al., 2014). The prevalence of potential smartphone addiction was 18.1%, similar to the range reported in the Kwon et al. (2013) for Korean adolescents (16.6% in boys, 26.6% in girls).

In this study, participants reported that, on average, 43.87% of their smartphone use was for leisure, 45.94% for learning, and 9.59% for work, indicating the percentage of total daily smartphone use devoted to leisure and learning was similar in adolescents. Thus, Hypothesis 1 was supported. These results are inconsistent with previous studies that have shown the majority of smartphone use was for leisure in college students (70–88% use for leisure) (Lepp et al., 2013, 2017; Barkley and Lepp, 2016) and adults (61% use for leisure) (Fennell et al., 2019). However, the purpose of smartphone use was significantly different between SAG and non-SAG, namely, the SAG had greater smartphone use for leisure, while the non-SAG had greater smartphone use for learning. These results combined with previous logistic regression analysis results showed that levels of smartphone addiction were reduced when smartphones were used for learning (Lee et al., 2017), suggesting that parents and teachers should provide guidance for adolescents about specific functions of smartphone use, such as learning or searching for information to reduce smartphone addiction.

Regarding situational use, 90.9% of all adolescents reported using the smartphone primarily while sitting. This is very similar to previous research in samples of college students (87%) and adults (81%) (Barkley and Lepp, 2016; Fennell et al., 2019). It seems that smartphones, despite their portability and mobility, are primarily sedentary devices for all individuals regardless of age. So Hypothesis 2 was verified.

Congruent with previous studies (Barkley and Lepp, 2016; Fennell et al., 2019), our finding showed that sedentary behavior was negatively correlated with use time on smartphones, suggesting that individuals who allocated more time for daily sitting use smartphones for greater periods. But beyond the time on smartphone use of previous studies, our results demonstrated that sedentary behavior was also negatively associated with smartphone addiction, indicating that our findings more comprehensively revealed the relationships between sedentary behavior and problematic smartphone use. Thus, Hypothesis 3 was supported. In addition, prior studies have identified sedentary behavior as an independent risk factor for cardiovascular disease (Katzmarzyk et al., 2009; Carter et al., 2017), which is worrisome as individuals with smartphone addiction spent more time on sedentary behavior and are at greater risk for cardiovascular disease than those without smartphone addiction.

Unlike sedentary behavior, the results of relationship between physical activity and problematic smartphone use were inconsistent in prior studies. For example, some researchers found there was no direct relationship between volume of daily physical activity and time on smartphone use (Barkley and Lepp, 2016; Fennell et al., 2019), while Kim et al. (2015) revealed that average number of walking steps per day negatively correlated with smartphone addiction. Other researches demonstrated that using the smartphone for texting during treadmill exercise may reduce participation in vigorous intensity exercise (Rebold et al., 2016), while using the smartphone for listening to music has been shown to increase exercise intensity (Rebold et al., 2015), suggesting the relationship between physical activity and smartphone depending on the aspect of smartphone functions. Based on the results from previous studies, we speculate that smartphone use may increase sedentary behavior by using traditional forms of screen-based apps while simultaneously prompt physical activity by using health related apps.

Novel to our study was our demonstration that not only the relationships between sedentary behavior and time spent on smartphone use but also between sedentary behavior and smartphone addiction were moderated by self-control. These two associations were stronger for individuals with low self-control

than for those with high self-control. Therefore, Hypothesis 4 was verified, which indicates the sense of moderation of self-control in the relationship between sedentary behavior and problematic smartphone use. These findings are consistent with recent theorizing on the trait of self-control (de Ridder et al., 2012; Hofmann et al., 2014b) and prior studies (Cooper et al., 2017; Liu et al., 2018b) indicating the protective role of self-control. Individuals who are high in self-control are more likely to reduce problematic smartphone use even though their sedentary behavior is at a high level. Presumably, these individuals have developed a good coping strategy and self-control capacities that help them to avoid using the smartphone when they are sitting. In contrast, smartphone use among persons who are low in self-control seems to be more strongly influenced by sedentary behavior. An explanation could be that these individuals' attention was generally hijacked by the smartphone, which leads individuals to respond immediately to smartphone signals when they are sitting (Berger et al., 2018).

LIMITATIONS AND IMPLICATIONS

Several limitations of the present study are noteworthy. First, due to the cross-sectional survey design in this study, causal relationships between sedentary behavior and problematic smartphone use should be interpreted with great caution. Future research may adopt longitudinal or experimental study models to strictly identify the causal relationships among these variables. Second, due to social desirability and other biases, the self-report method might inflate shared method variance and restrict the validity of the data. Future research using objective methods (such as ActiGraph accelerometers and smartphone apps) to assess the sedentary behavior and smartphone use may be necessary to address this. Third, self-control can be subdivided into trait self-control and state self-control; however, only the trait self-control was considered in our study, potentially limiting the utilization of the present study. Future research should try to investigate both state and trait self-control.

Despite the above limitations, the results of this study contribute to an expanding of the scope of interventions geared toward preventing problematic smartphone use in adolescents. Our data show that sedentary behavior was negatively correlated with problematic smartphone use. Although we cannot determine causal relationships between sedentary behavior and problematic smartphone use, reducing sedentary behavior is undoubtedly beneficial for alleviating problematic smartphone use. In fact, China's government has enacted a "National Teenagers' Sunny Sports Program" with the goal of having students do 1 h of exercise every day to promote physical activity and reduce sedentary behaviors. We speculate that such a program is not only useful in the field of physical fitness but also in curbing excessive or problematic smartphone use. Other interventions could target adolescents with low self-control by raising their awareness of their tendency to problematic smartphone use and launching evidence-based public health programs for improving self-control levels. Fortunately, promising results have been found in prior research on self-control training (Hofmann et al., 2014a; Friese et al., 2017). One big advantage of such trainings may be the high domain-general capacity; training in self-control in one field may lead to broad improvements in other fields over time. For example, Zou et al. (2016) have found that participating in 5 weeks of aerobic exercise (physical self-control) can increase self-control after ego-depletion in terms of pain tolerance. These pieces of evidence give reason to assume that adolescents low in self-control could benefit from exercise training, leading them to reduced problematic smartphone use.

CONCLUSION

This study has found that the majority of smartphone use was for leisure and learning, which was positively associated with sedentary behavior in Chinese adolescents. Furthermore, results of this study provided evidence that self-control exerts a moderating role on the impact of sedentary behavior on adolescents with problematic smartphone use. In other words, strengthening self-control may be effective in helping adolescents with sedentary behavior to limit their problematic smartphone use. The current study expands the pediatric literature on sedentary behavior and problematic smartphone use during the potentially critical developmental period of adolescence and points to the need to launch evidence-based exercise interventions and self-control training for adolescents at risk for problematic smartphone use.

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 the Human Experimental Ethics Board of Guangzhou Sports University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

M-QX, LL, and MH contributed to the conception and design of the study. Z-RW and JL organized the database. M-QX and LL analyzed the data. M-QX and Z-RW wrote the first draft of the manuscript. MH and ZX contributed to the manuscript revision, read, and approved the submitted version.

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Memory Function and Brain Functional Connectivity Adaptations Following Multiple-Modality Exercise and Mind–Motor Training in Older Adults at Risk of Dementia: An Exploratory Sub-Study

Narlon C. Boa Sorte Silva[1,2], Lindsay S. Nagamatsu[1], Dawn P. Gill[2,3,4], Adrian M. Owen[5] and Robert J. Petrella[1,2,3,4,6,7]**

[1] School of Kinesiology, Faculty of Health Sciences, Western University, London, ON, Canada, [2] Centre for Studies in Family Medicine, Department of Family Medicine, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada, [3] Graduate Program in Health and Rehabilitation Sciences, Faculty of Health Sciences, Western University, London, ON, Canada, [4] School of Health Studies, Faculty of Health Sciences, Western University, London, ON, Canada, [5] The Brain and Mind Institute, Department of Physiology and Pharmacology, and Psychology, Western University, London, ON, Canada, [6] Canadian Centre for Activity and Aging, Western University, London, ON, Canada, [7] Department of Family Practice, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada

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***Correspondence:**
Narlon C. Boa Sorte Silva
nboasort@uwo.ca
Robert J. Petrella
petrella@uwo.ca

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Background: Multiple-modality exercise improves brain function. However, whether task-based brain functional connectivity (FC) following exercise suggests adaptations in preferential brain regions is unclear. The objective of this study was to explore memory function and task-related FC changes following multiple-modality exercise and mind–motor training in older adults with subjective cognitive complaints.

Methods: We performed secondary analysis of memory function data in older adults [n = 127, mean age 67.5 (7.3) years, 71% women] randomized to an exercise intervention comprised of 45 min of multiple-modality exercise with additional 15 min of mind-motor training (M4 group, n = 63) or an active control group (M2 group, n = 64). In total, both groups exercised for 60 min/day, 3 days/week, for 24 weeks. We then conducted exploratory analyses of functional magnetic resonance imaging (fMRI) data collected from a sample of participants from the M4 group [n = 9, mean age 67.8 (8.8) years, 8 women] who completed baseline and follow-up task-based fMRI assessment. Four computer-based memory tasks from the Cambridge Brain Sciences cognitive battery (i.e. Monkey Ladder, Spatial Span, Digit Span, Paired Associates) were employed, and participants underwent 5 min of continuous fMRI data collection while completing the tasks. Behavioral data were analyzed using linear mixed models for repeated measures and paired-samples t-test. All fMRI data were analyzed using group-level independent component analysis and dual regression procedures, correcting for voxel-wise comparisons.

Results: Our findings indicated that the M4 group showed greater improvements in the Paired Associates tasks compared to the M2 group at 24 weeks [mean difference: 0.47, 95% confidence interval (CI): 0.08 to 0.86, p = 0.019]. For our fMRI

analysis, dual regression revealed significant decrease in FC co-activation in the right precentral/postcentral gyri after the exercise program during the Spatial Span task (corrected $p = 0.008$), although there was no change in the behavioral task performance. Only trends for changes in FC were found for the other tasks (all corrected $p < 0.09$). In addition, for the Paired Associates task, there was a trend for increased co-activation in the right temporal lobe (Brodmann Area = 38, corrected $p = 0.07$), and left middle frontal temporal gyrus (corrected $p = 0.06$). *Post hoc* analysis exploring voxel FC within each group spatial map confirmed FC activation trends observed from dual regression.

Conclusion: Our findings suggest that multiple modality exercise with mind–motor training resulted in greater improvements in memory compared to an active control group. There were divergent FC adaptations including significant decreased co-activation in the precentral/postcentral gyri during the Spatial Span task. Borderline significant changes during the Paired Associates tasks in FC provided insight into the potential of our intervention to promote improvements in visuospatial memory and impart FC adaptations in brain regions relevant to Alzheimer's disease risk.

Clinical Trial Registration: The trial was registered in ClinicalTrials.gov in April 2014, Identifier: NCT02136368.

Keywords: functional connectivity, functional magnetic resonance imaging, memory, multiple-modality, mind–motor, exercise, cognitive training, older adults

INTRODUCTION

Findings from laboratory work and clinical trials for the treatment of dementias, such as Alzheimer's disease, have consistently produced disappointing results, with the possibility of a single cure being very unlikely (Mangialasche et al., 2010; Sperling et al., 2011). Efforts have been made to identify and intervene with those who are at greater risk of cognitive decline and dementia before the establishment of clinical impairment (Jessen et al., 2014b). Older adults with subjective cognitive complaints (SCC) (Jessen et al., 2014a; Slot et al., 2018) may represent a portion of the population experiencing early signs of cognitive decline due to underlying pathophysiological changes before clinical impairment is obvious (Chen et al., 2014; Buckley et al., 2015). The focus on preclinical stages of dementia has included the impact of preventive measures such as exercise and cognitive training years prior to disease onset (Livingston et al., 2017). If prevention programs could delay the onset of dementia even in part of the at-risk population, this could decrease the disease prevalence significantly (Brookmeyer et al., 2007; The Alzheimer Society of Canada in collaboration with the Public Health Agency of Canada, 2016). Healthy lifestyle choices, including exercise, may be an important strategy to prevent or slow the progression of dementia in the aging population (Barnes et al., 2003; Alzheimer Society of Canada, 2010; Livingston et al., 2017), even in those with high genetic risk (Lourida et al., 2019).

Exercise has been associated with preserved age-related cognitive functioning in observational studies (Barnes et al., 2003; Abbott et al., 2004; Weuve et al., 2004; Bugg and Head, 2011; Bugg et al., 2012) and improved cognition (Lautenschlager et al., 2008), as well as positive functional (Voss et al., 2010; Chirles et al., 2017) and structural (Erickson et al., 2011) brain changes in longitudinal interventional studies. The positive effects of exercise on behavioral and neuroimaging outcomes in older adults are well documented, but less is known about the effects of exercise in brain functional connectivity (FC). Brain FC can be understood as temporal and functional correlations of spatially distinct cortical and subcortical structures active at rest and/or during task in blood oxygenation level-dependent (BOLD) functional magnetic resonance imaging (fMRI) (Buckner et al., 2009, 2013). Intrinsic FC data consist of anatomically and/or functionally distinct neuronal networks underlying neural function, particularly necessary for higher-order cognitive processes (Buckner et al., 2009, 2013). From a clinical perspective, FC can also aid in the identification of neurodegenerative processes occurring early on in the spectrum of dementia. For instance, Song et al. (2015) reported on resting-state FC disruption in the medial temporal lobe associated with Alzheimer's disease biomarker deposition in cognitively healthy older adults (Song et al., 2015). Others have postulated that resting-state FC disruption in the default mode network (DMN) is evident in Alzheimer's disease patients compared to healthy controls (Greicius et al., 2004; Schwindt et al., 2013), which is also pronounced in individuals with mild cognitive impairment (MCI), along with changes in the medial temporal lobe (MTL) network, prior to Alzheimer's disease diagnosis (Sorg et al., 2007).

Exploring changes in FC in older adults at risk of Alzheimer's disease and dementia is, therefore, imperative. Of particular interest, previous resting-state fMRI studies have shown that exercise might impart positive effects in enhancing FC in resting-state networks in healthy individuals and in those with

MCI (Voss et al., 2010; Chirles et al., 2017). These studies have primarily focused on the effects of aerobic exercise (AE) on FC changes within the DMN and MTL networks in healthy and MCI patients, due to the clinical implications of these networks in the context of Alzheimer's disease (Greicius et al., 2004; Sorg et al., 2007; Schwindt et al., 2013). Despite promising research with resting-state FC studies, less is known on the effect of multiple-modality exercise on task-related FC in older adults at risk of dementia. Focusing on task-related FC could aid in understanding the influence of exercise in FC underlying neurocognitive processes in those at higher risk of dementia. In addition, as we progress toward more comprehensive interventions that impart improvements to overall health in older adults, it is of interest to investigate whether multiple-modality exercise training (e.g. AE, resistance, or balance training), along with cognitively engaging tasks (i.e. mind–motor training), could have a different impact on FC in these individuals beyond traditional AE alone (Ngandu et al., 2015). Unfortunately, very few studies have explored the effects of combining different exercise modalities (i.e. multiple-modality exercise) and mind–motor training in brain functional and/or structural outcomes (Callisaya et al., 2017; Ji et al., 2017; Rehfeld et al., 2018; Teixeira et al., 2018). Only a short-term (6 weeks), quasi-experimental study included FC as an outcome with results indicating increased FC between the posterior cingulate cortex with cingulate, temporal, parietal, and occipital regions in the multiple-modality exercise group compared to a control group (Ji et al., 2017). Because of limited evidence, further research is warranted.

Square-stepping exercise (SSE) (Shigematsu et al., 2008) is a novel form of mind–motor training, which has been associated with positive effects on global and domain-specific cognitive functioning in older adults (Gill et al., 2016; Boa Sorte Silva et al., 2018b). Although the impact of SSE on cognitive function remains relatively unknown, evidence suggests the potential for SSE to benefit cognition, especially by improving memory (Teixeira et al., 2013; Shigematsu, 2014). Our group has investigated the effects of SSE in cognition, mobility, and oculomotor function in older adults with and without cognitive impairment (Gill et al., 2016; Heath et al., 2017; Boa Sorte Silva et al., 2018a,b). Nevertheless, the effects of SSE on task-related FC remain to be determined.

Therefore, the objective of this exploratory study was to investigate changes in memory function in a group of older adults following multiple-modality exercise with mind–motor training compared to multiple-modality exercise alone. Further, we investigated task-related FC changes in memory in a subsample of older adults with SCC derived from our full randomized controlled trial (RCT) (Boa Sorte Silva et al., 2018b).

MATERIALS AND METHODS

Study Design

Our study design, recruitment, and inclusion criteria have been reported previously (Boa Sorte Silva et al., 2018b). This study is a secondary analysis of memory function outcomes from our full RCT as well as an exploratory study involving a subsample of individuals who underwent fMRI assessment at baseline and 24 weeks. Participants in the experimental group were randomized to a 24-week intervention [*m*ultiple-*m*odality exercise and *m*ind–*m*otor training (M4 group)] targeted at improving cognitive function, mobility, and cardiovascular health (Gregory et al., 2016). Participants in the control group received an active control intervention [multiple-modality exercise plus balance, range of motion, and breathing exercise (M2 group)]. A subsample of participants from the experimental arm (M4 group) underwent fMRI assessment at baseline and 24 weeks later. The study was registered with ClinicalTrials.gov in April 2014 (Identifier: NCT02136368). The Western University Health Sciences Research Ethics Board approved this project, and all participants provided written informed consent prior to taking part in the study.

Participants

For this secondary analysis of memory function, we examined data from 127 participants, while for the exploratory fMRI study, we examined fMRI data from nine participants who completed both baseline and 24-week assessments. As applied in our full trial (Gregory et al., 2016), the study included community-dwelling individuals aged 55 years or older with self-reported SCC (defined as answering positively to the question "Do you feel like your memory or thinking skills have got worse recently?") (Barnes et al., 2013), and with preserved instrumental activities of daily living (Gregory et al., 2016). In addition to the full trial inclusion criteria, only right-handed participants were included in this sub-study. Individuals with a diagnosis of dementia and/or scoring <24 on the Mini-Mental State Examination (MMSE) (Gregory et al., 2016), history of stroke or transient ischemic attacks, or presented with MRI contraindications were also excluded.

Exercise Intervention

The exercise experimental protocol has been published previously (Gregory et al., 2016). Briefly, participants in the M4 group received a 45-min multiple-modality exercise program (i.e. aerobic training and resistance training) with an additional 15 min of mind–motor training (i.e. SSE). Participants in the active control group received 24 weeks of multiple-modality exercise with additional balance, range of motion and breathing exercises. For both arms of the study, sessions were administered in groups of less than 25 participants, 60 min/day, 3 days/week, for 24 weeks.

Multiple-Modality Exercise

The multiple-modality exercise intervention incorporated a 5-min warm-up, a 20-min AE, a 5-min cool down, followed by 10 min of resistance training and 5 min of stretching. AE intensity was prescribed via target heart rates (HR) determined at baseline using the STEP^{TM} tool (Stuckey et al., 2012). During the AE component, participants were encouraged to keep their HR at 65–85% of their predicted maximum HR (HRmax) and/or at a rating of 5–8 on the 10-point modified Borg Rating of Perceived Exertion (RPE) scale (Chodzko-Zajko et al., 2009).

HR monitoring was conducted part way through and at the end of the AE component during each exercise session. Participants were instructed to record the HR and RPE immediately after each monitoring in a training log provided by the research team. Target HR were recalculated at 12 weeks to adjust for progression in the AE training.

Mind–Motor Training

The SSE program is a group-based intervention performed on a gridded floor mat (2.5 × 1 m) containing 10 rows with four equal-sized squares per row. The training protocol entails the reproduction of previously demonstrated complex stepping patterns on the SSE mat (see **Figure 1**). The stepping patterns are demonstrated by an instructor, and participants are expected to memorize and further attempt to reproduce each stepping pattern by memory. Instructors could not physically intervene, but in instances where participants were having difficulty reproducing the SSE patterns, they were provided oral cues. There are more than 200 stepping patterns created for SSE (Shigematsu et al., 2008), and the complexity of these stepping patterns is given according to the number of steps per pattern, as well as the order and direction of foot placement across the SSE mat. In our study, the SSE sessions were carried out in groups of no more than six participants per mat. To ensure equal group progression throughout the program, the complexity of the stepping patterns within each session was increased only when the majority of participants (i.e. 75%) had successfully performed a given stepping pattern at least four times. The goal was to progress through as many SSE patterns as possible over the 24-week intervention period. Additionally, to create a positive social atmosphere, participants were encouraged to assist each other, as necessary, by providing cues to accurately perform the stepping patterns.

fMRI Data Collection

Participants were invited to attend a 1-h fMRI session at the Robarts Research Institute at Western University. Image acquisition was performed in a Siemens MAGNETOM Fit whole-body 3 Tesla MRI scanner with in-plane acceleration (GRAPPA = 2). Structural MR images (T1-weighted anatomical images) were acquired for each participant lying passively in the magnet with the following parameters: echo time (TE): 2.98 ms, repetition time (TR): 2,300 ms, time for inversion (TI): 900 ms, and flip angle = 9°, field of view (FOV) = 256 mm, voxel size: 1 × 1 × 1 mm. Whole-brain, task-related functional imaging was performed using a gradient-echo echoplanar imaging (EPI) sequence (36 slices) sensitive to the BOLD contrast with the following parameters: TE: 30 ms, TR: 2,000 ms, flip angle = 70°, FOV = 240 mm, voxel size: 3 × 3 × 3 mm.

FIGURE 1 | Participants performing stepping patterns during a square-stepping exercise session.

The procedure allowed us to acquire 145 functional MR images over 5 min of continuous data collection, while the participants were presented with each cognitive task. Tasks were displayed on a projector screen, visible from the bore of the MRI scanner via a mirror. In each task, participants were required to click on the screen to select their answers using an MRI-compatible tracker ball mouse. The tasks were programmed in the Adobe Flex development environment and were administered as a stand-alone software within the Adobe Integrated Runtime (AIR) environment. The study experiment consisted of a design-free, data-driven approach where a specific design (e.g. block or event-related) was not established (Poldrack et al., 2008; Huettel, 2012). The tasks used have been adapted from tests used in previous neuroimaging and patient studies at our institution (Owen et al., 2010; Hampshire et al., 2012). Tasks were behaviorally piloted by volunteers prior to scanning in order to ensure optimal performance for generating fMRI contrasts of interest (i.e. BOLD). The general approach used for task design was standardized across all four memory tasks described in the subsequent sections.

Behavioral Tasks

The four cognitive tasks were administered in this study at baseline and 24 weeks and were derived from the Cambridge Brain Sciences (CBS) computerized cognitive battery (Hampshire et al., 2012). Although we collected data from 12 cognitive tasks within the CBS cognitive battery, for this secondary analysis, we decided to focus only on four memory tasks, namely, Monkey Ladder, Spatial Span, Digit Span, and Paired Associates. We had data available from 127 participants at baseline, collected over 2 days using a computer laptop; see our published protocol for more details (Gregory et al., 2016). The rationale to focus on these memory tasks is based on the fact that for our full RCT, the memory composite derived from these four tasks showed trends for greater changes following the 24-week exercise program and showed significant changes 56 weeks after baseline assessments (Boa Sorte Silva et al., 2018b). However, data from each individual task, as well as the fMRI data, have not yet been published. Below is the description of each individual task:

(a) Monkey Ladder is based on a task from the animal literature (non-human primates) and assesses working memory ability (Inoue and Matsuzawa, 2007). In this task, sets of numbered boxes are displayed all at the same time at random locations within a grid. After a variable interval

(number of boxes multiplied by 900 ms), the numbers are removed leaving just the blank boxes visible. Participants are requested to respond by clicking on the boxes in ascending numerical sequence. The difficulty of the task is modulated as follows: the number of boxes presented increases by one if the participant answers correctly and decreases by one if the participant makes a mistake. The outcome measure is the length of the longest sequence successfully remembered.

(b) Spatial Span is a task to measure spatial short-term memory capacity in humans (Kessels et al., 2000). In this task, 16 boxes are displayed in a grid. A sequence of randomly selected boxes flashes one at a time at a rate of 900 ms per box. Subsequently, a tone cues the participant to repeat the sequence by clicking on the boxes in the same order in which they flashed. The difficulty of the task is modulated as follows: the number of boxes that flash increases by one if the participant answers correctly and decreases by one if the participant makes a mistake. The outcome measure is the length of the longest sequence successfully remembered.

(c) Digit Span is based on the verbal working memory component of the WAIS-R intelligence test (Wechsler, 1981). In this task, participants view a sequence of digits that appear on the screen one at a time. Subsequently, participants are required to repeat the sequence of numbers using the mouse cursor to click a series of numbered buttons that appear along the bottom of the screen. The difficulty of the task is modulated as follows: the sequence of numbers on the screen increases by one if the participant answers correctly and decreases by one if the participant makes a mistake. The outcome measure is the length of the longest digit sequence successfully remembered.

(d) Paired Associates is a visuospatial paired associate learning task (Gould et al., 2005). In this task, boxes are displayed at random locations on a grid. The boxes open one after another to reveal an enclosed icon, after which they close. Subsequently, the icons are displayed in random order in the center of the grid, and the participant must click on the boxes that contained them. The difficulty of the task is modulated as follows: if the participant remembers all the icon–location pairs correctly, then the next trial will have one more box. If a mistake is made, the next trial has one less box. The outcome measure is the length of the longest sequence successfully remembered.

Behavioral Data Analysis

All behavioral data collected from our full sample were analyzed using linear mixed models for repeated measurements (Fitzmaurice et al., 2011) to assess differences between groups in mean change from baseline to 24 weeks. In the models, we also examined differences within groups from baseline to 24 weeks. The terms included in the models were group, time, and group × time interaction. Time was modeled categorically using two indicator variables representing each time point (baseline as reference category). Task scores were z transformed. All analyses were performed using the intent-to-treat approach, including all randomized participants, regardless of compliance with the program and follow-up assessments (Fitzmaurice et al., 2011). Behavioral data collected during fMRI image acquisition in our exploratory analysis were analyzed via paired-samples *t*-tests in SPSS®. We also calculated Cohen's *d* for paired-samples *t*-tests at *post hoc* using the formula $d = t/\sqrt{n}$, where *d* corresponds to Cohen's *d*, *t* represents *t*-scores, and *n* is the sample size (Lakens, 2013). Analysis of behavioral data was done in order to inform and contextualize the results from fMRI data.

FMRI Data Analysis

All data analysis was performed using FMRIB's Software Library (FSL) tools.[1] *Post hoc* analysis was performed in SPSS® for Mac, Version 21 (Armonk, NY, United States). The study pipeline for image acquisition and data analysis is illustrated in **Figure 2**.

Preprocessing

Structural images were brain extracted using an in-house script and inspected for optimal extraction. Functional images were registered using FLIRT linear registration to each individual's structural image and then a 2-mm MNI template registration. We then applied motion correction, brain extraction, spatial smoothing (5-mm FWHM Gaussian kernel) and high-pass temporal filtering (Jenkinson and Smith, 2001; Jenkinson et al., 2002).

Processing

Functional data analysis was performed using probabilistic independent component analysis (Beckmann and Smith, 2004) as implemented in FSL's Multivariate Exploratory Linear Decomposition into Independent Components (MELODIC) Version 3.15 (Hyvärinen, 1999; Minka, 2000; Beckmann and Smith, 2004). At the subject level, MELODIC results were decomposed into independent components that represent large-scale patterns of functional network connectivity using independent component analysis (ICA). Individual-level ICA maps were inspected to identify components that were considered noise using a visually inspected structured artifact removal approach (i.e. hand removal) (Griffanti et al., 2017), as previously applied in a similar exercise study (Rajab et al., 2014). All independent components that were identified as noise were removed from individual-level data via spatial regression using FSL's *fsl_regfilt* tool. These components were composed of noise due to several sources such as head motion, cerebral spinal fluid signal, respiratory and cardiac rhythms, scan parameters, and others.

Main Analysis

Following individual-level MELODIC, we then performed group-level ICA to identify independent components that represent large-scale patterns of FC within the group-level spatial maps, and the independent components were set at 40 per task, based on inspection of individual-level ICA results to inform optimal fitting of the data. Results from group-level MELODIC were further analyzed using FSL's dual regression tool. In this approach, the set of spatial maps from the group-average analysis

[1] www.fmrib.ox.ac.uk/fsl

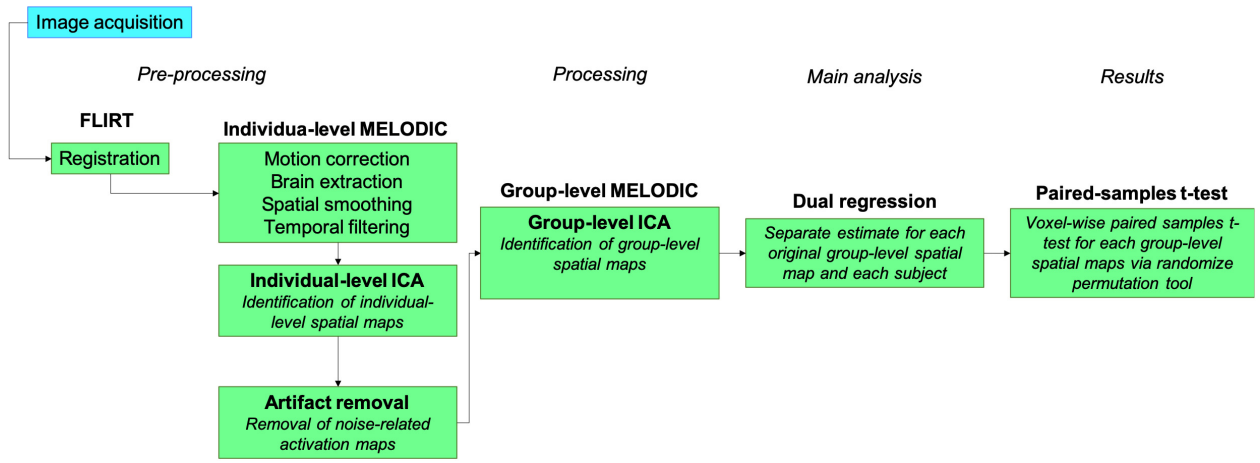

FIGURE 2 | fMRI data analysis pipeline.

was used to generate subject-specific versions of the spatial maps, and associated time series (Nickerson et al., 2017). Primarily, for each individual in the study, the group-average set of spatial maps is regressed (as spatial regressors in a multiple regression) into the subject's 4D space–time dataset; this results in a set of subject-specific time series, one per group-level spatial map. Later, those time series are regressed (as temporal regressors in a multiple regression) into the same 4D dataset, resulting in a set of subject-specific spatial maps, one per group-level spatial map. This procedure ultimately unfolds in a separate estimate for each original group-ICA map and each subject. In the final step of our analysis, we then performed paired-samples *t*-tests in a voxel-wise analysis for each of the group-level spatial maps using FSL's randomize permutation-testing tool (5,000 permutations, threshold-free cluster enhancement) corrected for voxel-wise multiple comparisons. Our goal was to identify any significant changes in the group-level spatial maps from baseline to 24 weeks. If any changes were identified, our results could indicate that the exercise program might have imparted adaptations in FC.

Post hoc Analysis

We further performed *post hoc* analysis using subject-specific spatial maps (stage 2 outputs from dual regression) to quantify changes in the strength of connectivity within a group-level spatial map from baseline to 24 weeks, following previous methodology (Glahn et al., 2010). To accomplish this, we used the group-level spatial map as binary network masks and calculated an index that would indicate, on average, how strongly the voxels within a group-level spatial map are related to each other for each individual (via FSL's *fslmeants*). We were interested in knowing whether this FC index would have changed following the exercise program (Glahn et al., 2010; Rajab et al., 2014). We also performed a similar procedure to quantify the changes in specific regions that showed significant changes from baseline to 24 weeks in the main analysis. Instead of using a binary mask, this was accomplished by extracting a voxel connectivity index from the exact location where changes from baseline to 24 weeks occurred (i.e. using MNI152 coordinates in *fslmeants*); the coordinates were defined based on significant or borderline significant results of dual regression. The indices calculated as a result of these procedures were then analyzed in a paired-samples *t*-test in SPSS®.

RESULTS

Details regarding study enrolment, randomization, and adherence have been reported elsewhere (Boa Sorte Silva et al., 2018a,b). Briefly, 169 individuals were assessed for eligibility, 11 did not meet the inclusion criteria, and 31 declined to participate. Thus, 127 participants were included and randomized to either the M2 ($n = 64$) or M4 ($n = 63$) groups; 109 participants attended assessments at 24 weeks. Demographic characteristics for our full sample are shown in **Table 1**. For our fMRI exploratory study, the sample was composed of mostly females who were approximately 70 years of age and with a Montreal Cognitive Assessment (MoCA) score of approximately 25, suggesting the presence of objective cognitive impairment in addition to the self-reported SCC but with no indication of dementia (mean MMSE score of 29) (Nasreddine et al., 2005). Participant demographic and clinical characteristics for this subsample are presented in **Supplementary Table 1**.

Behavioral Results

For our full sample ($n = 127$), the M4 group showed greater improvements in the Paired Associates tasks compared to the M2 group at 24 weeks [mean difference: 0.47, 95% confidence interval (CI): 0.08 to 0.86, $p = 0.019$] (see **Table 2**), which resulted from an improvement in the M4 group from baseline to 24 weeks ($p = 0.001$), while changes in the M2 group were not observed ($p = 0.93$). Participants in both groups showed improvements in the Monkey Ladder task ($p \leq 0.01$); however, there were no differences between groups at follow-up. No within- or between-group changes were observed for the Spatial Span and Digit Span

TABLE 1 | Baseline characteristics of study participants by randomization group.

Variables†	M4 (*n* = 63)	M2 (*n* = 64)
Demographics		
Age, year	67.6 (7.5)	67.4 (7.2)
Women	44 (69.8%)	46 (71.9%)
Caucasian	61 (96.8%)	62 (98.4%)
Education, year	13.3 (2.7)	13.8 (3)
MoCA, score	25.3 (2.7)	25.6 (2.4)
MMSE, score	29 (1.2)	29.2 (1)
Weight, kg	80 (13.8)	80.8 (17.7)
Height, m	1.65 (0.1)	1.65 (0.1)
BMI, kg/m^2	29 (4.1)	29.7 (6.2)
Medical history, *n* (%)		
Hypertension	36 (57.1%)	32 (50%)
Hypercholesterolemia	28 (44.4%)	23 (35.9%)
Type 2 diabetes	7 (11.1%)	5 (7.8%)
Myocardial infarction	5 (7.9%)	4 (6.3%)
Atrial fibrillation	3 (4.8%)	–
Angina/coronary artery disease	2 (3.2%)	1 (1.6%)
Aneurysm	2 (3.2%)	1 (1.6%)
Former smoker	29 (46%)	28 (44.4%)
Current smoker	1 (1.6%)	1 (1.6%)
Memory Tasks, *z* scores		
Monkey Ladder	0.05 (1.03)	−0.05 (0.97)
Spatial Span	−0.04 (1.05)	0.04 (0.95)
Digit Span	−0.1 (1.03)	0.28 (1.75)
Paired Associates	−0.09 (0.95)	0.09 (1.05)

†*Data presented either as mean (standard deviation) or no. (%) where applicable. M2, multiple-modality group; M4, multiple-modality, mind–motor group; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; BMI, body mass index.*

tasks; however, the M4 group showed trends for improvements in the Digit Span task ($p = 0.06$).

For our subsample of participants in the fMRI exploratory study ($n = 9$), the results indicated no significant differences from baseline to 24 weeks in all of the tasks studied. For the Paired Associates task, however, we observed a trend for significant differences compared to baseline for the task max score [mean difference: 0.75, 95% CI: −0.1 to 1.6, $t(7) = 2.05$, $p = 0.08$, Cohen's $d = 0.72$] and task mean score [mean difference: 0.4, 95% CI: −0.1 to 0.8, $t(7) = 0.08$, Cohen's $d = 0.74$], corroborating the results from our full sample. The results are presented in **Supplementary Table 2**.

fMRI Results

Group-level ICA via MELODIC identified several independent components across all four tasks; one component included previously studied networks such as the DMN (**Supplementary Figure 1**). Considering the exploratory nature of the study, we investigated significant and borderline significant changes across all independent components identified across all four tasks. Dual regression results indicated significant change in FC after the 24-week program within only one of the group-level spatial maps in the Spatial Span task and overall borderline significant changes in eight other regions in the brain, of which seven were further explored and one was excluded as it was considered not relevant for the purposes of this study. The results for each task are reported in further detail below, except for the Monkey Ladder task as no differences were observed at post-test.

For the Spatial Span task across all 40 group-level spatial maps [i.e. Spatial Span-independent components (SS)], dual regression revealed significantly decreased co-activation in the right precentral/postcentral gyri (MNI: 36, −22, 58) after the exercise program within SS16 (corrected $p = 0.008$), as shown in **Figure 3A**. There were also borderline significant differences suggesting an increased co-activation in the left frontal orbital cortex [(MNI: −36, 27, −22), corrected $p = 0.08$], with participants showing increased activation at post-test compared to baseline within SS06 (please see **Figure 3B**). Similarly, borderline significantly decreased co-activation in the left frontal lobule/superior frontal gyrus [(MNI: −18, 42, 31), Brodmann Area (BA) 9, corrected $p = 0.09$] within SS23, as shown in **Figure 3C**. Additionally, a borderline increased co-activation was seen following the exercise program in the left occipital fusiform gyrus/lateral occipital cortex [(MNI: −40, −74, −16), BA 19, corrected $p = 0.07$] within SS30, as shown in **Figure 3D**. The brain regions identified to be involved in each independent component for the Spatial Span task are reported in **Table 3**.

For the Digit Span task, there were no significant differences following the exercise program across all 40 group-level spatial maps [i.e. Digit Span-independent components (DS)]. However, borderline significant differences were found in the DS06 in which increased co-activation was seen in the left occipital fusiform gyrus [(MNI: −40, −68, −22), corrected $p = 0.08$]

TABLE 2 | Within- and between-group differences from baseline to 24 weeks by randomization group.†

	Within-group differences (95% CI)				Between-group differences (95% CI)	
Outcomes	**M4 (*n* = 63)**	***p*-value**	**M2 (*n* = 64)**	***p*-value**	**24 weeks (*n* = 127)**	***p*-value**
Monkey Ladder	**0.23 (0.05 to 0.41)**	**0.01**	**0.29 (0.12 to 0.47)**	**0.001**	−0.07 (−0.32 to 0.19)	0.6
Spatial Span	−0.07 (−0.25 to 0.12)	0.47	0.04 (−0.14 to 0.22)	0.67	−0.11 (−0.36 to 0.15)	0.42
Digit Span	0.33 (−0.02 to 0.69)	0.06	−0.06 (−0.4 to 0.29)	0.75	0.39 (−0.1 to 0.88)	0.12
Paired Associates	**0.48 (0.2 to 0.76)**	**0.001**	0.01 (−0.26 to 0.28)	0.93	**0.47 (0.08 to 0.86)**	**0.019**

†*Calculated from linear mixed effects regression models that included group (M4 or M4), time (baseline and 24 weeks), and group × time interaction terms. A total of four models were conducted—corresponding to each memory task listed in the first column. Results are represented as intent-to-treat approach. Bold numbers indicate significant differences within- or between-groups where applicable. M2, multiple-modality group; M4, multiple-modality, mind–motor group.*

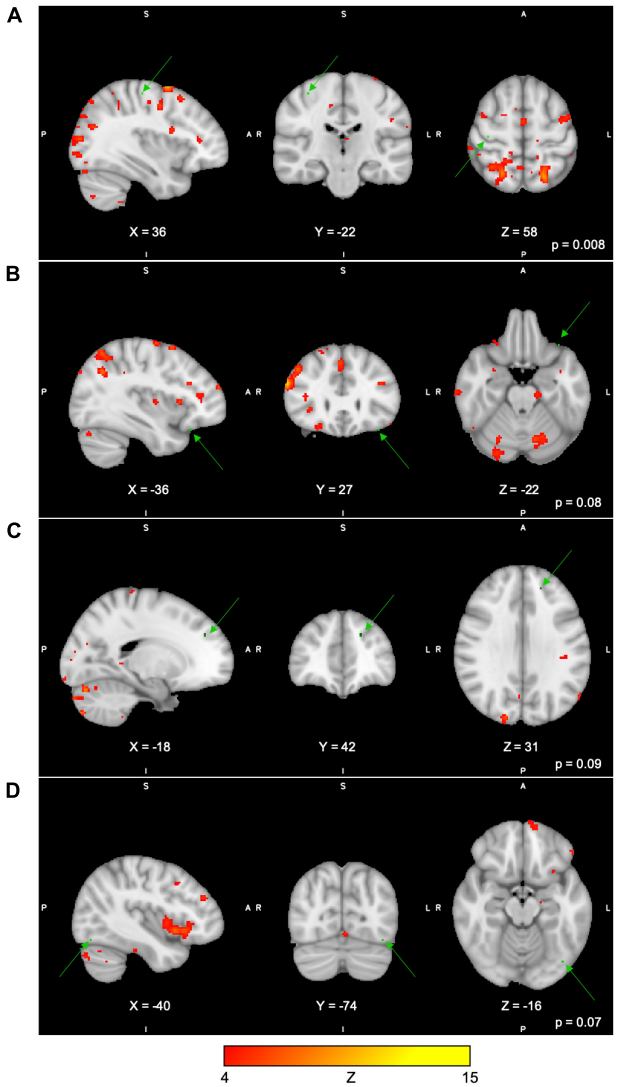

FIGURE 3 | (A–D) Changes within group during Spatial Span task. In red–yellow contrast are Spatial Span-independent components 16 **(A)**, 06 **(B)**, 23 **(C)**, and 30 **(D)**. In dark–light green are regions with changes in group-level spatial map co-activation after the exercise program (green arrows).

(see **Figure 4A**). In addition, increased co-activation was seen within the DS08 located in the left inferior temporal gyrus [(MNI: −48, −10, −32), corrected $p = 0.09$] (see **Figure 4B**). The brain regions identified to be involved in each independent component for the Digit Span task are reported in **Table 4**.

For Paired Associates task across all 40 group-level spatial maps [i.e. Paired Associates-independent components (PA)], there were no significant differences following the exercise program. However, borderline significant differences were found in PA15 in which increased co-activation was seen in the right temporal lobe [(MNI: 46, 18, −40), BA 38, corrected $p = 0.07$], as well as in PA34, where decreased co-activation was seen in the left middle temporal gyrus [(MNI: −60, −32, −8), corrected $p = 0.06$] following the exercise program (please see **Figures 5A,B**). The brain regions identified to be involved in each independent component for the Paired Associates task are reported in **Table 5**.

TABLE 3 | Brain regions composing the Spatial Span-independent components (group-level spatial maps) identified via independent component analysis.

Brain regions	MNI coordinates (*x*, *y*, *z*)	*Z* score
SS06		
Cerebellum	23, −36, −32	11.3
Fontal lobule (BA 10), R	5, 57, 34	9.1
Inferior frontal gyrus, R	56, 28, 25	13.2
Lateral occipital cortex, R	56, −63, −14	10.1
Middle frontal gyrus, R	50, 33, 33	15.1
Precentral gyrus, R	51, 10, 30	13.9
Precentral gyrus, L	−52, −0, 50	9.9
Precuneus cortex, L	−2, −78, 42	10.5
Supramarginal gyrus, R	59, −40, 44	12.5
SS16		
Angular gyrus, L	−43, −53, 19	10.7
Inferior frontal gyrus, R	48, 8, 15	10.1
Lateral occipital cortex, L	−23, −89, 13	11.5
Lateral occipital cortex, R	56, −60, 13	13.4
Middle frontal gyrus, R	33, 5, 65	13.1
Occipital pole, R	18, −98, 7	12.6
Precentral gyrus, L	−44, −2, 35	11.2
Superior frontal gyrus, R	22, −6, 74	10.9
Superior parietal lobule, L	−24, −54, 55	10.3
SS23		
Cerebellum	42, −51, −49	14.3
Lateral occipital cortex, R	12, −63, 64	9.4
Postcentral gyrus, R	28, −37, 75	9.8
SS30		
Central opercular cortex, L	−47, 3, 3	9.8
Inferior frontal gyrus, L	−57, 22, 14	9.4

SS, Spatial Span-independent components (group-level spatial maps); BA, Brodmann area; L, left hemisphere; R, right hemisphere. Regions are reported as peak of cluster activation (Z score) within each component.

Post hoc Analysis

In our *post hoc* analysis (using dual regression stage 2 outputs), we explored within group-level spatial map by extracting summary values that indicated how strongly the voxels of a given map were associated with the time course for that map (e.g. Spatial Span 16) and whether those values changed over time. This *post hoc* analysis was limited to group-level spatial maps that were significant in our main analysis (i.e. dual regression). We extracted summary values from the entire group-level spatial maps as well as for the specific locations that showed changes over time using MNI coordinates. For example, we looked at the average connectivity change within the right precentral/postcentral gyri (MNI: 36, −22, 58) for SS16 from baseline to 24 weeks.

Our results indicated that there were no significant changes in group-level spatial maps average FC from baseline to 24 weeks across all three tasks. When only considering the regions where significant or borderline significant changes occurred in the main

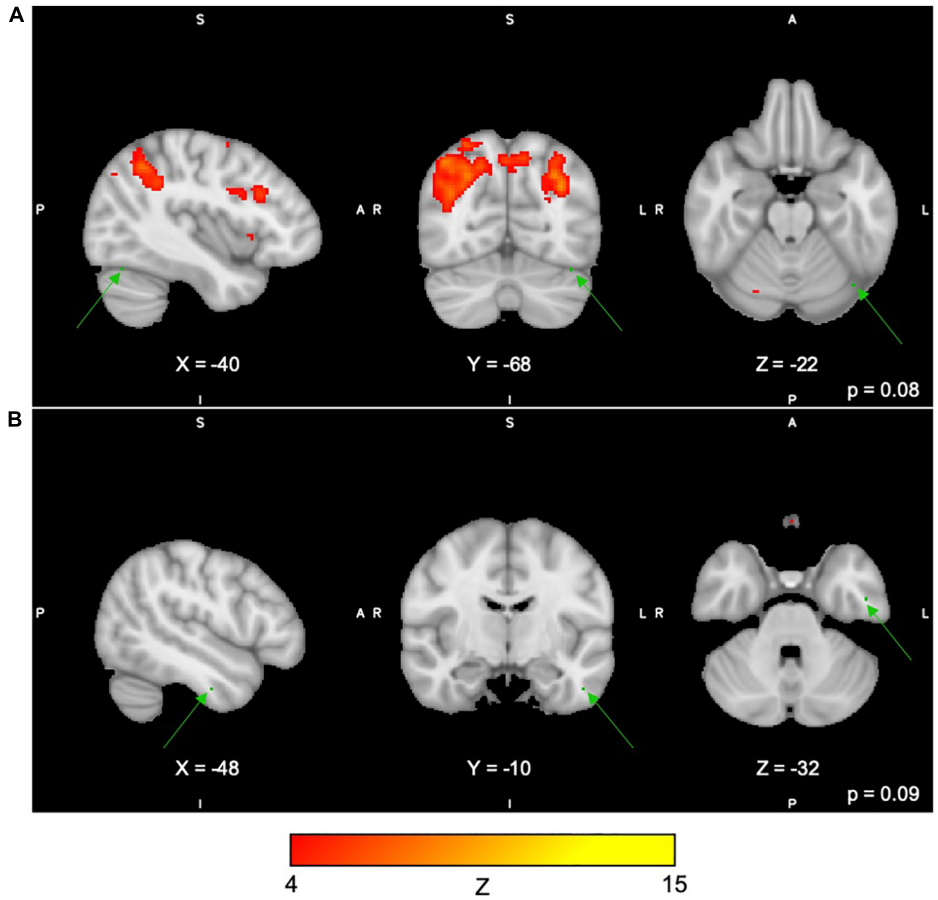

FIGURE 4 | (A,B) Changes within group during the Digit Span task. In red–yellow contrast are Digit Span-independent components 06 **(A)** and 08 **(B)**. In dark–light green are regions with changes in group-level spatial map co-activation after the exercise program (green arrows).

TABLE 4 | Brain regions composing the Digit Span-independent components (group-level spatial maps) identified via independent component analysis.

Brain regions	MNI coordinates (*x*, *y*, *z*)	*Z* score
DS06		
Supramarginal gyrus, R	51, −44, 43	9.7
Supramarginal gyrus, L	−45, −49, 42	9.7
Superior parietal lobule, L	−31, −55, 44	13.2
Lateral occipital cortex, R	31, −64, 58	9.9
Lateral occipital cortex, L	−24, −60, 44	12.3
Angular gyrus, R	45, −57, 44	12.2
DS08		
Subcallosal cortex, L	−10, 23, −17	11.9
Frontal medial cortex, R	10, 34, −20	12.5
Frontal medial cortex, L	−7, 38, −17	10.8

DS, Digit Span-independent components (group-level spatial maps); L, left hemisphere; R, right hemisphere. Regions are reported as peak of cluster activation (Z score) within each component.

analysis, we noted changes in the average FC from baseline to 24 weeks, which confirmed the results from dual regression. The results are summarized in **Figures 6**, **7**.

DISCUSSION

We conducted a secondary analysis of four memory tasks following a 24-week multiple-modality exercise and with or without additional mind–motor training. We also conducted a data-driven exploratory analysis of task-related cortical FC changes as a result of multiple-modality exercise and mind–motor training (M4 group) in older adults with SCC at increased risk for dementia. Following 24 weeks of intervention, we observed significant differences between groups in the Paired Associates tasks, favoring the experimental group, which received additional mind–motor training (i.e. M4 group) compared to the active control group. Further, our exploratory analysis revealed significant and borderline significant changes in FC during three of the four memory tasks administered in our study. Owing to the approach used in our investigation, the results from our fMRI substudy must be interpreted within the context of each task and each independent component derived from the ICA. Our analysis was aimed at exploring within-group spatial map FC changes after the intervention. Using MELODIC ICA, we were able to identify independent components that included brain regions that were temporally associated (i.e. co-activation) during

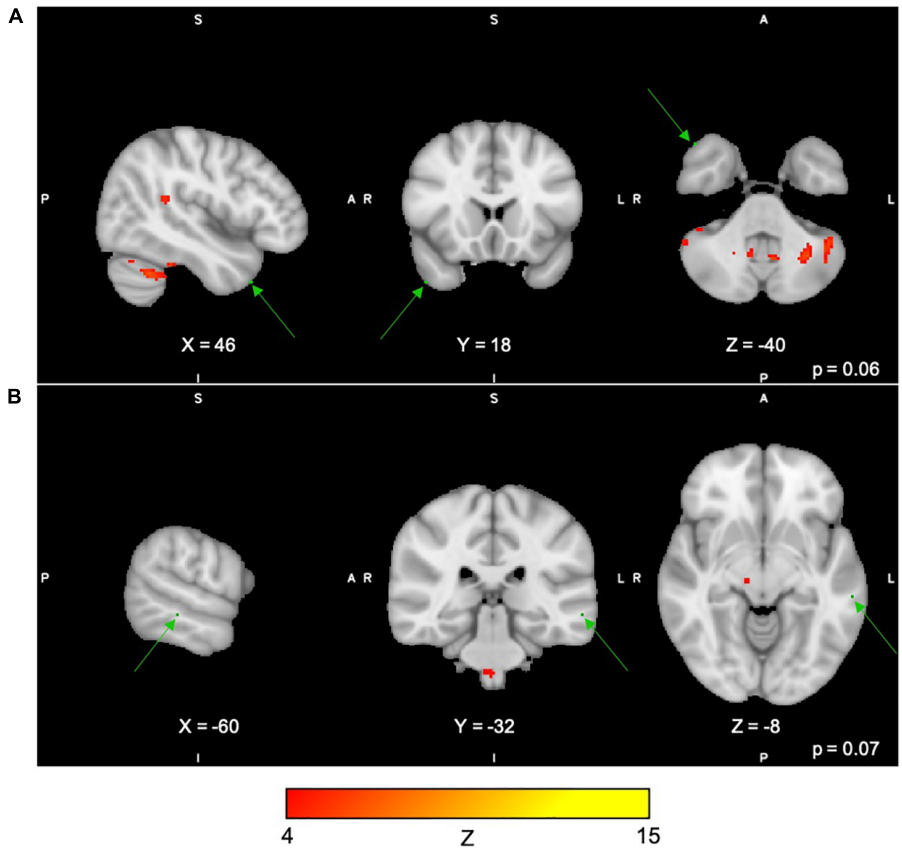

FIGURE 5 | (A,B) Changes within group during the Paired Associates task. In red–yellow contrast are IC15 **(A)** and IC08 **(B)**. In dark–light green are regions of decreased co-activation after the exercise program (green arrows).

each task and, therefore, could be understood as functionally associated (Dosenbach et al., 2007; Biswal et al., 2010). It is relevant to note that some of the regions that also co-active during a task (temporally, but not functionally correlated) might not necessarily be a result of task-related processes, but rather the result of other neuronal processes concurrent to task performance (Hampson et al., 2006). With these considerations, it is then possible to question whether the intervention had any impact within the FC of the brain for a given task in our study.

Overall, the results from our full sample suggested that additional mind–motor training yielded greater changes in memory measured in the Paired Associates task superior to multiple-modality exercise without mind–motor training, with trends for significant changes in the Digit Span task at the follow-up. Results from our exploratory, data-driven fMRI analysis indicated that our experimental condition might have imparted divergent effects on cortical FC across the tasks employed; however, the results must be considered with caution. More specifically, for the Spatial Span task, we observed decreased co-activation in the precentral/postcentral gyri (corrected $p = 0.008$) and left frontal lobe/superior frontal gyrus (trend), as well as increased co-activation in the left frontal orbital cortex and left occipital fusiform gyrus/lateral occipital cortex (trend). For the Digit Span task, we observed increased co-activation in the left occipital fusiform gyrus and left inferior temporal gyrus (trend). Last, for the Paired Associates task, we observed increased co-activation in the right temporal lobe (trend) and decreased co-activation in the left middle temporal gyrus (trend). Our *post hoc* analysis investigating changes in FC strength across the entire group-level spatial maps following previous methodology (Glahn et al., 2010) revealed no significant differences following the program. Although, when exploring each specific cortical region

TABLE 5 | Brain regions composing the contrast are shown Paired Associates-independent components (group-level spatial maps) identified via independent component analysis.

Brain regions	MNI coordinates (*x*, *y*, *z*)	*Z* score
PA15		
Cerebellum	−30, −51, 43	17.4
Lingual gyrus, L	−6, −52, −2	10.7
Supramarginal gyrus, R	53, −41, 20	9.7
Middle temporal gyrus, R	52, −48, 6	9.2
PA34		
Temporal pole (BA21), L	−25, 3, −36	8.2
Parahippocampal gyrus, L	−24, 1, −36	8.1

PA, Paired Associates-independent components (group-level spatial maps); L, left hemisphere; R, right hemisphere. Regions are reported as peak of cluster activation (Z score) within each component.

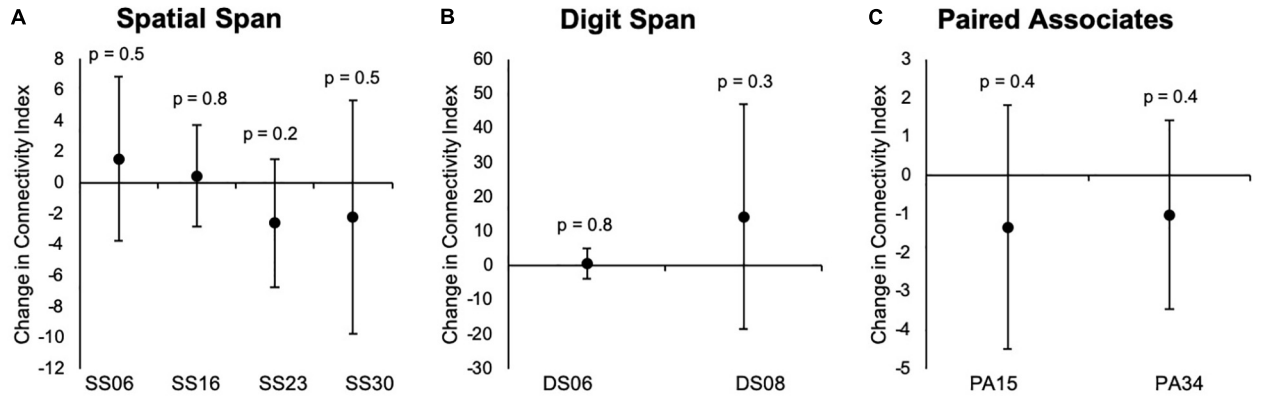

FIGURE 6 | (A–C) Changes overtime in the average strength of functional connectivity within each group-level spatial maps for each task that showed significant changes from baseline to 24 weeks in the main analysis. Data are presented as mean difference from baseline to 24 weeks and associated confidence interval, along with *p*-values for significant changes. SS, Spatial Span-independent components; DS, Digit Span-independent components; PA, Paired Associates-independent components.

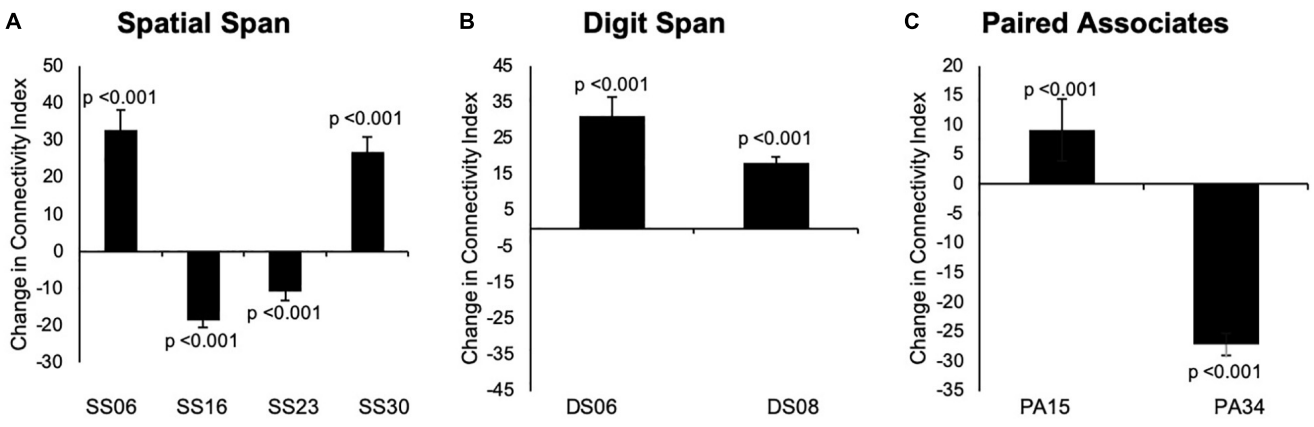

FIGURE 7 | (A–C) Changes overtime in the average strength of functional connectivity for the specific region where changes from baseline to 24 weeks in group-level spatial map co-activation were observed. Graph A illustrates changes in the left frontal orbital cortex (MNI: –36, 27, –22) for SS06; right precentral/postcentral gyri (MNI: 36, –22, 58) for SS16, left frontal lobule/superior frontal gyrus (MNI: –18, 42, 31, BA 9) for SS23, and left occipital fusiform gyrus/lateral occipital cortex (–40, –74, –16, BA 19) for SS30. Graph B illustrates changes in the left occipital fusiform gyrus (MNI: –40, –68, –22) for DS06 and left inferior temporal gyrus (MNI: –48, –10, –32) for DS08. Graph C illustrates changes in the right temporal lobe (MNI: 46, 18, –40, BA 38) for PA15 and left middle temporal gyrus (MNI: –60, –32, –8) for PA34. Data are presented as mean difference from baseline to 24 weeks and associated confidence interval, along with *p*-values for significant changes. Note: SS, Spatial Span; DS, Digit Span; PA, Paired Associates-independent components; MNI, Montreal Neurological Institute coordinates; BA, Brodmann area.

within the group-level spatial maps for connectivity strength, we encountered statistical significance, suggesting a confirmation of the changes in the co-activation in the spatial maps (please see **Figure 7**).

Across all four tasks, significant changes were seen only for the Spatial Span task in the right precentral/postcentral gyri. For this task, we observed a decreased co-activation within the group-level spatial maps from baseline to 24 weeks. The group-level spatial map (SS16) in which this change occurred involves co-activation of brain regions previously associated with executive control (e.g. superior parietal lobule), working memory (e.g. superior frontal gyrus), as well as sensorimotor and visuospatial areas (Shirer et al., 2012). In the context of this group-level spatial map, it is possible to suggest that the decreased FC of the precentral/postcentral gyri with the other cortical regions did not have an imperative effect on task performance at 24 weeks, owing to the fact that there were no significant changes in the behavioral scores for the Spatial Span task for our full sample, nor for our subsample in this M4 group.

The Spatial Span task is believed to measure spatial short-term memory ability (Kessels et al., 2000). It is noteworthy that our program included a 15-min block of SSE, in which participants are expected to memorize and reproduce increasingly complex stepping patterns on a gridded floor map (Shigematsu et al., 2008). Arguably, the SSE program demands increased attention and short-term spatial memory recall, which could lead to improvements in overall spatial memory performance. Although speculative, it is possible that the SSE program, in addition to the multiple-modality exercise program, could have yielded FC changes involving co-activation of the precentral/postcentral gyri

during Spatial Span task performance in the current study. This could be further supported by trends of increased co-activation observed in the left inferior temporal gyrus during another task in this study, the Digit Span task, a region engaged in motor function and known to show decreased connectivity in older adults compared to young individuals in resting-state fMRI (Voss et al., 2013). Because of methodological limitations, these interpretations must be interpreted with caution.

It is, however, undoubtedly challenging to attribute changes in FC of motor-related regions (i.e. precentral/postcentral gyri and left inferior temporal gyrus) during computer-based memory tasks to the effects of our program, since we are unable to establish a direct connection between changes in the co-activity and task performance, in addition to estimating region engagement from resting to task-related states (Hampson et al., 2006). Because of a lack of significant changes in the behavioral measures for the Spatial Span and Digit Span tasks (trend for significant changes in the full sample), it is also difficult to suggest whether increases in co-activation would indicate negative changes in FC due to aging or disease-related processes and/or whether decreases in co-activation would indicate efficiency during task performance due to the intervention applied in our study. Moreover, as mentioned above, these processes could also be considered task irrelevant, which might or might not be detrimental to task performance (Hampson et al., 2006). In addition, a previous study did not observe changes in FC of the motor regions following 6 and 12 months of AE in older adults (Voss et al., 2010). Voss et al. (2010) reported that the exercise program did not lead to any changes in regional FC in motor areas such as the right precentral gyrus and left inferior temporal gyrus. There is evidence from animal literature suggesting brain plasticity identified as increased synaptic density and expression of proteins associated with dendritic growth in motor-related regions following treadmill exercise (Swain et al., 2003; Ferreira et al., 2010), and even more so with more complex motor training (Garcia et al., 2012).

Therefore, in our limited design, we cannot determine with certainty if the task-related FC changes observed in our study are due to the intervention itself and whether these are positive meaningful changes. In the context of previous studies adopting a similar data analysis methodology, Chirles et al. (2017) investigated FC changes in older adults diagnosed with MCI following a 12-week AE program (Chirles et al., 2017). The authors were mainly interested in exploring FC of the posterior cingulate cortex and precuneus within the DMN. The authors reported an increased co-activation in resting-state FC between the posterior cingulate cortex and precuneus regions and the several other cortical regions, including the right postcentral gyrus. This suggested that the aerobic program enhanced recruitment of preserved brain regions in MCI patients, which possibly reflected in improvements in behavioral measures of cognitive function. The FC improvements were not seen in the healthy control group—despite improvements in behavioral measures in these participants (Chirles et al., 2017).

Noteworthy, we reported borderline significant changes (confirmed in our *post hoc* region-specific analysis) in FC in the right temporal lobe (BA 38) and left middle temporal gyrus during the Paired Associates task, two regions heavily involved in memory processes (Shirer et al., 2012). Moreover, our behavioral data showed greater changes in the Paired Associates tasks for our full sample analysis and also borderline significant changes in the task performance in our subsample. Under these considerations, we can postulate that our multiple-modality exercise and mind–motor training program might have had a positive effect in FC underlying visuospatial memory, as measured by improved performance in the full sample, and in our nine participants from the M4 group (trend at $p = 0.08$) in the Paired Associates task with a medium-to-large effect size (i.e. Cohen's d for max score = 0.72 and 0.74 for mean score) (**Supplementary Table 2**). More importantly, the results from our full sample analysis revealed that there were indeed significant improvements in the Paired Associates task performance above and beyond the active control group ($p = 0.001$ for changes overtime and $p = 0.019$ for difference between groups at 24 weeks). The data from our full sample offers confirmation and strengthens our borderline significant changes in the Paired Associates behavioral data within our subsample, which can then provide context and assist in interpretation of the borderline significant changes in FC observed in this fMRI sub-study.

Cortical regions involved in the group-level spatial maps where the FC changes occurred, that is, independent components PA15 and PA34 (please see **Table 5**), were predominately located in the medial temporal lobe, including the left and right hippocampi, parahippocampal gyri, and middle temporal gyrus (please see **Supplementary Figures 2A,B**). It is well-known that these regions have been implicated in memory function (Alvarez and Squire, 1994; Jeneson and Squire, 2011; Shirer et al., 2012), and have been observed to be heavily involved in the Paired Associates task memory encoding and retrieval (De Rover et al., 2011). From a clinical perspective, these findings could have important implications, considering that these aforementioned regions are hallmarks of pathophysiological changes (e.g. amyloid beta deposition) in MCI and early/prodromal stages of Alzheimer's disease (Taylor and Probst, 2008), including cortical atrophy proceeding Alzheimer's disease diagnosis (Pettigrew et al., 2017), and disruption of resting-state FC, possibly due to Alzheimer's disease biomarker deposition (Song et al., 2015). Moreover, the performance on a variant of the Paired Associates task employed in this study demonstrated marked differences between MCI patients and healthy controls, characterized by decreased bilateral hippocampal and parahippocampal activation during tasks in MCI patients compared to controls (De Rover et al., 2011).

Here, we were able to demonstrate significant changes in the behavioral component of memory function measured via the Paired Associates task. This is an encouraging result, and future research could investigate the effects of multiple-modality exercise and mind–motor training in medial temporal lobe regions, employing a full RCT design and including resting-state and task-related FC as the main outcomes. It would be relevant to use a task such as the Paired Associates task to explore such effects, as postulated by De Rover et al. (2011), regarding the relevance of the task as a possible biomarker of Alzheimer's disease risk (De Rover et al., 2011).

Limitations

Our findings should be interpreted with caution and in the context of our limitations. Although the CBS is grounded in well-validated neuropsychological tests (Hampshire et al., 2012), this is the first study to apply this method to evaluate the effects of exercise in memory function in older adults with SCC. Also, participants included in this study were predominantly Caucasian, well educated, and functionally independent; thus, our results may not be generalizable to other populations. For our exploratory fMRI substudy, our data analysis was restricted to nine subjects only, a very small sample size, limiting our ability to generalize the results. We had limited resources to collect fMRI data from our active control group, and therefore, we cannot establish certainty on whether our findings were due to the main effects of the intervention program—even though the results from the experimental group in our full data analysis of memory tasks showed greater changes in memory following the program (driven by changes in the Paired Associates task), superior to the active control group. In addition, we did not include resting-state data in our study, impairing our ability to determine which regions identified in the task-derived independent components were, in fact, relevant to the task performance or were a result of other processes irrelevant to the task performance (Hampson et al., 2006). Importantly, our group-level results were also susceptible to artifacts, and the group-level maps could have included regions in which co-activation was seen due to noise, despite our efforts to correctly identify and remove artifact-driven independent components at the individual level. In addition, despite our efforts to mitigate sources of noise and variability influencing the BOLD response, we acknowledge that this is still a possibility. However, it is unlikely that the individual-level and group-level maps would significantly suffer from, or be heavily influenced by, the variability of BOLD response or non-task processes, as BOLD variability would be a product of random noise, and not a specific pattern equally present in all individuals during assessment.

Another limitation of our study was that we adopted a model-free approach to analyze the task-related fMRI data (Rogers et al., 2007). We used this approach to investigate whole-brain, voxel-wise FC maps that could have been active for the duration of each task, and therefore, our methods were restricted to data-driven exploratory analysis as opposed to a hypothesis-driven approach (where previous knowledge would have informed the decision of limiting analysis to a set of cortical and subcortical regions of interest) (Rogers et al., 2007). In addition, our model-free design only allowed us to collect data during a single 5-min block of ongoing trials for each task, and consequently, we were not able to time lock the stimulus and data collection of each single trial within the block (as commonly done in event-related or block design studies). This could have ultimately reduced our power to detect true significant treatment effects (Huettel, 2012).

In addition, FC data is particularly sensitive to head motion and physiological artifacts linked to respiratory and cardiac rhythms (Buckner et al., 2013). Furthermore, the FC data provide essential insights into the cortical and subcortical coupling at rest and during task-related fMRI; however, it is unknown whether the observed FC within a group-level spatial map in this study reflects stable or temporary connectivity configurations in the brain (Buckner et al., 2013). Finally, our study employed a multi-domain intervention, involving components of aerobic training, resistance training, as well as mind–motor training. This is a novel approach, and to our knowledge, no previous neuroimaging studies have been conducted to investigate changes in FC during memory tasks in older adults with SCC following a multi-domain program such as ours. Therefore, methodological differences between our study and previous studies create a barrier to draw conclusions regarding our results.

CONCLUSION

Our aim was to explore the effects of 24 weeks of multiple-modality exercise with or without additional mind–motor training in four memory tasks, and explore task-related, cortical and subcortical FC changes in older adults with SCC. Our findings indicated that multiple-modality exercise with additional mind–motor training yielded greater changes in memory function during the Paired Associates task compared to an active control group. Further, our intervention might have resulted in divergent FC adaptations, including significant decreased co-activation in the precentral/postcentral gyri during the Spatial Span task. Of particular interest, we also reported borderline significant increased co-activation in the right temporal lobe, accompanied by a decreased co-activation in the left middle temporal gyrus within the two group-level spatial maps involving regions of the medial temporal lobe during the Paired Associates task. These findings provide insight into the potential of our multiple-modality exercise and mind–motor training intervention to promote improvements in behavioral measures of visuospatial memory, as well as impart FC adaptations in brain regions relevant to Alzheimer's disease risk. Future research should emphasize the clinical relevance of these FC changes following exercise in the context of disease prevention and treatment.

AUTHOR'S NOTE

This work was conducted at the Parkwood Research Institute, in affiliation with the Lawson Health Research Institute and St. Joseph's Health Care (London, ON, Canada).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Western University Health Sciences Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NB was responsible for the study design, data preprocessing and analysis, interpretation of results, and drafting of the final manuscript. LN was responsible for the data preprocessing and analysis, interpretation of results, and reviewing and editing the manuscript. DG was responsible for the study design, data collection, and reviewing of the manuscript. AO was responsible for the study design and reviewing and editing the manuscript. RP acquired funding for the study, was responsible for the study design, data collection, interpretation of results, and reviewing and editing 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.2020.00022/full#supplementary-material

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Memory Traces Diminished by Exercise Affect New Learning as Proactive Facilitation

Cuicui Li[1], Rena Li[2,3] and Chenglin Zhou[1]**

[1] Department of Sport Psychology, School of Sport Science, Shanghai University of Sport, Shanghai, China, [2] Beijing Key Laboratory of Mental Disorders, Beijing Anding Hospital, Capital Medical University, Beijing, China, [3] Beijing Institute for Brain Disorders, Capital Medical University, Beijing, China

Exercise enhances cognitive function through increased neurogenesis but can also cause neurogenesis-induced forgetting. It remains unclear whether the diminished memory traces are completely forgotten. Our goals were to determine whether spatial memory is diminished by exercise, and if so, whether the memory is completely gone or whether only the local details disappear but not the acquired strategy. Two-month-old male C57BL/6J mice were trained on a spatial memory task using the Morris water maze and tested to determine that they had learned the platform location. Another mouse group received no training. Half the mice in each group then exercised on a running wheel, while the other half remained sedentary in home cages. After 4 weeks of this, previously trained mice were tested for their retention of the platform location. All mice were then subjected to the task, but the platform was located in a different position (reversal learning for previously trained mice). We found that exercise significantly facilitated the forgetting of the first platform location (i.e., diminished spatial memory) but also significantly enhanced reversal learning. Compared with mice that received no pre-exercise training, mice that had been previously trained, even those in the exercise group that had decreased recall, showed significantly better performance in the reversal learning test. Activation of new adult-born neurons was also examined. Although newborn neuron activation between groups that had or had not received prior task training was not different, activation was significantly higher in exercise groups than in sedentary groups after the probe test for reversal learning. These results indicated that the experience of pre-exercise training equally facilitated new learning in the sedentary and exercise groups, even though significantly lower memory retention was found in the exercise group, suggesting rule-based learning in mice. Furthermore, newborn neurons equally participated in similar and novel memory acquisition.

Keywords: exercise, forgetting, reversal learning, proactive facilitation, adult-born neurons

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****Correspondence:***
Rena Li
renali@ccmu.edu.cn
Chenglin Zhou
Chenglin_600@126.com

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INTRODUCTION

Although neurogenesis is one of the primary mechanisms whereby exercise benefits cognitive enhancement, neurogenesis-induced forgetting has also been widely shown (Akers et al., 2014; Gao et al., 2018; Ladron de Guevara-Miranda et al., 2018; Ishikawa et al., 2019). This kind of forgetting was discovered in the phenomenon of "infantile amnesia." As we all experienced, it is hard for an adult to recall experiences that happened during their infancy, and memories

during infancy are more fragile than those during adulthood. Animal studies gave the evidence that infantile rodents had poorer memory retention than adult ones, and this different ability in memory retention was related to the number of newborn neurons in the dentate gyrus (DG) of the hippocampus (Akers et al., 2014; Guskjolen et al., 2016). Hippocampal DG is one of the brain regions maintaining continuous newborn neurons generation after animals matured (Alvarez-Buylla and Lim, 2004). The number of adult-born neurons in DG can be enhanced by the external environment, such as enriched environment and exercise (Nokia et al., 2016; Diederich et al., 2017; Sakalem et al., 2017; Tharmaratnam et al., 2017). Studies showed that exercise could also facilitate the forgetting of previous memory traces through neurogenesis in adult mice, and when exercise-induced neurogenesis is blocked using genetic techniques, exercise-induced forgetting is simultaneously diminished (Akers et al., 2014; Epp et al., 2016). However, numerous studies have shown that exercise could improve memory encoding and consolidation, and this beneficial effect was partially mediated by neurogenesis (Kronenberg et al., 2006; Ahlskog et al., 2011; Alomari et al., 2013; Pontifex et al., 2016; Sakalem et al., 2017). Studies from Epp et al. (2016) partially explained these conflicting results on exercise-induced effects on memories through neurogenesis. They suggested that neurogenesis-induced forgetting would benefit reducing proactive interference (PI) from previous memory traces, therefore further facilitate the following memory encoding with conflict information (Epp et al., 2016). It seems that neurogenesis facilitates the forgetting of remote memories and enhances recent memories. Consistent with this hypothesis, computer modeling studies to simulate the integration of newborn neurons have shown different contributions of neurogenesis to remote and recent memories and have suggested an increase in the forgetting of remote memories with an enhancement for the preservation of recent memories (Weisz and Argibay, 2012).

However, whether these diminished memory traces completely disappear is still unknown. Our knowledge of the external world is always based on the long-term memories we experienced previously. Human studies have shown the benefits of long-term memory for learning new information with similar principle (Gerven et al., 2017; Oberauer et al., 2017; Wang et al., 2017). In addition, rule-based learning (combined cues with an abstract principle) has been reported during associative learning both in human and animal studies (Racht-Delatour and Massioui, 1999; Wills et al., 2011; Maes et al., 2017; Broschard et al., 2019). Compared with remembering only separate cues, rule-based learning is more stable with the passing of time and can adjust to various contexts (Hoffmann et al., 2018). That is, the strategies to solve problems obtained from our experience are more reliable in memory and can facilitate subsequent new learning.

Therefore, in the present study, we hypothesized that the weakened memory traces induced by exercise would still benefit the subsequent new related learning by acting as an abstract strategy. In addition, adult-born neurons were suggested to be involved in both erasing remote and encoding recent memories. It is unclear whether there are any differences between the engagement of adult-born neurons in the acquisition of new memories with or without similar old memory traces. Thus, we further explored the engagement of adult-born neurons in a post-exercise spatial memory task (do or do not receive a pre-exercise spatial memory with similar paradigms). The newborn neurons were labeled with 5-bromo-2′-deoxyuridine (BrdU), and the participation of these neurons in memory acquisition was determined by labeling neurons with immediate early genes (IEGs), which were expressed after stimulation and have been widely used to assess the activation of neurons after memory tasks (Snyder et al., 2009; Belnoue et al., 2011).

MATERIALS AND METHODS

Animals and Experimental Design

The C57BL/6J male mice used in this study were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). All animals were housed in standard home cages in a room with constant temperature (22 ± 2°C) and humidity (50–60%) on a 12-h light/dark cycle (8:00-20:00). Food and water were available freely.

Mice were used in this study once they were 2 months old (young adults). All mice were handled 5 min each day for 5 days before experiments. To explore whether forgotten memory traces can affect new learning, animals were divided into four groups: (1) with memory traces (WMT) + exercise (Ex), (2) with no memory traces (NMT) + Ex, (3) WMT + sedentary (Sed), and (4) NMT + Sed. During pretesting, animals in the WMT groups were trained in a spatial reference memory task, the MWM, and acquired the spatial memory. Mice in the NMT groups stayed in their home cages, receiving no spatial memory training. Exercise (with sedentary controls) was conducted after the spatial reference memory task and lasted for 4 weeks. Mice in exercise groups were given a running wheel (ENV-044, Med-associates Inc) for voluntary running exercise. The amount of running revolutions were recorded continuously in a subset of cages. Each mouse ran an average of 6.89 km (±0.56 km) per day during the exercise period. After the exercise intervention, the memory retention of the mice in the WMT groups was evaluated on a probe test in the MWM task without the previously hidden platform in the pool. For the same operations among groups during post-exercise training, mice in the NMT groups were also placed in the maze and allowed to swim freely for 60 s. The next day, all mice were subjected to reversal training (Epp et al., 2016; **Figure 1**).

To label immature newborn cells, BrdU (100 mg/kg; Sigma) was injected intraperitoneally into mice in all groups twice daily for the last 4 days of the first week of exercise (**Figure 1**).

Morris Water Maze Task

Equipment

The water maze was a circular pool with a diameter of 120 cm. A platform was hidden in one quadrant of the pool 1 cm below the surface of the water, which was made opaque by the addition of milk. The pool was surrounded by a curtain having different shapes located on it in the east, south, west, and north areas. Mice

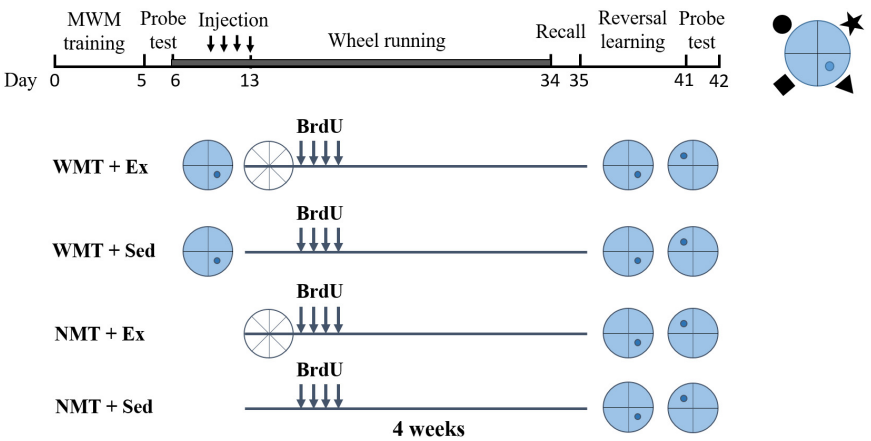

FIGURE 1 | Experimental timeline. Two groups of young adult mice with memory traces (WMT) were trained in the Morris water maze (blue circles with the platform location indicated in darker blue dot), whereas the other two groups with no memory traces (NMT) were not trained but remained in their cages. Half the mice were then subjected to 4 weeks of wheel running exercise (Ex; white circles) and the other half remained sedentary (Sed) in their home cage. To label newborn neurons, 5-bromo-2′-deoxyuridine (BrdU) was injected twice daily into all mice during the last 4 days of the first week of exercise. Following 4 weeks of exercise, a probe test was conducted in the Morris water maze to test recall, followed by a reversal learning task in the maze and a final probe test to examine new memory. A scheme of the position of the platform with respect to the cues used in Morris water maze was shown in the top right corner (the black shapes were the cues).

could use these shapes to determine where they were positioned in the maze and thus the location of the hidden platform once they found it. Animal behaviors were tracked and analyzed using ANY-maze software[1].

Pre-exercise Testing

The spatial reference memory task included 5 days of spatial navigation training and 1 day of probe testing. During spatial navigation training, mice learned to ascertain the position of the platform using the visual cues on the curtain during four trials conducted each day. Each trial was started by placing the mouse in one of the quadrants (in semi-random order) facing the wall of the pool. Four start locations were different during each day's training. And the start quadrant for each day was varied. During testing, the mouse was allowed to swim in the pool until it found the platform or for 60 s. When mice found the platform, they were allowed to stay on the platform for 15 s. If a mouse did not find the hidden platform after 60 s, it was guided to the platform and allowed to remain there for 15 s. Mice were given a probe test 24 h after the last day of training. During the probe test, the previously hidden platform was removed, and mice were allowed to swim for 60 s. The escape latency (the time it takes to find the platform), path length (the distance traveled to find the platform), and swimming speed were calculated during learning. The percent quadrant time (the percentage of time mice spent in a certain quadrant) in the target quadrant (TQ) and in the opposite quadrant (OQ), the average percent quadrant time spent in the two adjacent quadrants (AQs), and the quadrant crossings (the number of crossings in the aforementioned quadrants) during the probe test were collected. In addition, the latency to find the platform and the number of entries in the platform location were also examined.

[1]http://www.anymaze.co.uk

Post-exercise Testing

A probe test was conducted on the first day after 4 weeks of wheel-running exercise. This probe test was used to examine the ability of the mice to recall where the platform had been previously located in the pool. During this probe test, the latency to find the platform and the number of entries in the platform location, the percent quadrant time in the TQ and the OQ, the average percent quadrant time in the two AQs, and the quadrant crossings were examined. The next day, a reversal learning task was initiated. This task also consisted of 5 days of spatial navigation training and a probe test. The only difference between this training/testing and the pre-exercise training/testing was that the position of the hidden platform was moved to a different quadrant. For the 5-day navigation training during reversal learning, the escape latency, path length and swimming speed were collected. Similar to the measures described above, during the probe test of reversal learning, the platform latency and entries, the percent quadrant time, and the quadrant crossings were examined. For the analysis of the effect of memory traces on reversal learning, the original platform (platform during pre-exercise training) crossings and percent quadrant time in the TQ, AQs, and OQ were calculated for the WMT groups during reversal learning. The search strategies among the groups were investigated by examining the percent quadrant time in the corridor connecting the start position with the platform (before the first crossing of the platform location) during the reversal probe test.

Immunofluorescence

Mice were anesthetized 1.5 h after the end of behavior testing because IEGs are expressed 1-2 h after stimulation. The mice were perfused with 0.9% saline. One hemisphere of each mouse was fixed in 4% paraformaldehyde for 24 h and dehydrated for 30 h in a 30% sucrose solution for cytoprotection. The fixed tissue was then embedded in OCT. The hippocampus was

coronally sectioned using a cryostat microtome, with a section thickness of 40 μm.

Hippocampal tissue sections in one hemisphere were taken every 1 in a series of 6 sections (for double labeling) or every 1 in a series of 12 sections (for triple labeling) throughout the entire DG. Free-floating sections were placed in an antigen retrieval solution and heated in a water bath at 80°C for 30 min. The tissue was then placed in 1N HCl and incubated at 37°C for 30 min before being blocked in 3% normal goat serum (containing 0.5% Triton X-100) at room temperature for 1 h. Primary rat monoclonal BrdU antibody (1:300, Abcam, Cat# ab6326, RRID: AB_305426) and mouse monoclonal NeuN antibody (1:500, Millipore, Cat# MAB377, RRID: AB_2298772) with either rabbit anti-c-Fos antibody (1:200, Cell Signaling Technology, Cat# 2250, RRID: AB_2247211) or rabbit anti-EGR1 antibody (1:300, Cell Signaling Technology, Ca.# 4153, RRID: AB_2097038) were incubated with the tissue overnight at 4°C. The next day, the secondary antibodies goat anti-mouse Alexa Fluor 488 (1:500, Invitrogen), goat anti-mouse Alexa Fluor 594 (1:500, Invitrogen), or goat anti-mouse Alexa Fluor 647 (1:500, Invitrogen) were incubated as appropriate with the tissue for 1 h at room temperature to enable visualization of the primary antibodies.

The quantification of double/triple-labeled cells was performed using a confocal microscope (Zeiss, Jena, Germany) equipped with a 63 × oil-immersion objective. All potential labeled cells through the SGZ-GCL from multiple sections were scanned with a 1 μm interval. To estimate the total number of adult-born neurons for DG (bilaterally), the number of BrdU/NeuN immunoreactive cells was tallied and multiplied by 12 (Tronel et al., 2015). The proportion of the activated adult-born neurons was estimated by triple labeled by BrdU/NeuN/IEGs, and 100-200 adult-born cells were analyzed per mouse.

Statistical Analysis

All data are graphed as means ± standard error of the mean (SEM). Behavioral data, such as escape latency, path length, and swimming speed, during pre-exercise and reversal learning were analyzed using two-way or three-way repeated-measures analysis of variance (ANOVA). Behavioral parameters during the probe test, such as percent quadrant time and quadrant crossings, were analyzed using two-way ANOVA. The immunofluorescence data were analyzed using two-way ANOVA. The criterion for statistical significance was $\alpha = 0.05$. All analyses were carried out using SPSS, version 17.0 (StataCorp).

RESULTS

Exercise Reduces Memory Retention

Two-way repeated-measures ANOVA was used to analyze latency and path length during pre-exercise training. All mice in the WMT + Sed and WMT + Ex groups exhibited a similar ability to locate the platform during 5 days of learning (for latency: day main effect, $F_{(4,96)} = 48.387$, $p < 0.001$; group main effect, $F_{(1,24)} = 1.73$, $p > 0.05$; day × group interaction: $F_{(4,96)} = 0.557$, $p > 0.05$; for path length: day main effect: $F_{(4,96)} = 26.316$, $p < 0.001$; group main effect: $F_{(1,24)} = 4.061$, $p > 0.05$; day × group interaction: $F_{(4,96)} = 0.344$, $p > 0.05$) (**Figures 2A,B**). Swimming speeds between groups were examined using two-way repeated measures and the results showed increased swimming speeds for all mice during 5 days of water maze training, and no difference was found between groups (day main effect: $F_{(4,96)} = 5.645$, $p < 0.001$; group main effect: $F_{(1,24)} = 0.486$, $p > 0.05$; day × group interaction: $F_{(4,96)} = 0.584$, $p > 0.05$) (**Figure 2C**). During probe test, the percentage of time mice explored in different quadrants (TQ, AQs, and OQ) and quadrant crossings between groups were analyzed using two-way ANOVA. Mice in both groups showed significantly higher percentage time in the TQ compared with the other quadrants (quadrant main effect: $F_{(2,98)} = 7.817$, $p = 0.001$; group main effect: $F_{(1,98)} = 0.031$, $p > 0.05$; quadrant × group interaction: $F_{(2,98)} = 0.182$, $p > 0.05$) (**Figure 2D**). Similarly, mice in both groups performed a larger number of crossings in the TQ compared with the other quadrants (quadrant main effect: $F_{(2,98)} = 19.455$, $p < 0.001$; group main effect: $F_{(1,98)} = 3.077$, $p > 0.05$; quadrant × group interaction: $F_{(2,98)} = 0.127$, $p > 0.05$) (**Figure 2E**). The swimming speeds during probe test were also examined using Student's t test and no difference was found between the WMT + Sed and WMT + Ex groups ($t_{24} = 0.366$, $p > 0.05$) (**Figure 2F**).

To explore the effects of exercise on memory retention, we compared the latency to find the virtual platform and the number of crossings in the original platform location in the first probe test (platform removed) with that during the recall test (i.e., the probe test conducted after 4 weeks of wheel-running exercise) using a 2 (probe test: Pre-Ex, recall) × 2 (group: WMT + Sed, WMT + Ex) repeated-measures ANOVA. The results showed that 4 weeks' exercise intervention significantly increased the platform latency (probe test × group interaction: $F_{(1,24)} = 13.419$, $p = 0.001$; simple effects test: WMT + Ex group, $t_{12} = -5.127$, $p < 0.001$) and decreased the number of crossings in the platform location (probe test × group interaction: $F_{(1,24)} = 4.439$, $p < 0.05$; simple effects test: WMT + Ex group, $t_{12} = 2.999$, $p < 0.05$) (**Figures 3A–C**). We also compared the percentage of time mice searched in different quadrats (TQ, AQs, OQ) and the number of crossings in these quadrants between the WMT + Sed and WMT + Ex groups during recall. Two-way ANOVA was used in this analysis. The results showed that mice in the WMT + Ex group decreased the percentage of time spent in the TQ when compared with mice in the WMT + Sed group, while no difference was found among quadrants (quadrant × group interaction: $F_{(2,98)} = 3.469$, $p < 0.05$; simple effects test: WMT + Sed vs. WMT + Ex in TQ, $t_{24} = -2.478$, $p < 0.05$) (**Figure 3D**). In addition, mice in the WMT + Ex group exhibited fewer crossings in the TQ compared with mice in the WMT + Sed group. Mice in the WMT + Sed exhibited more entries in the TQ than the other quadrants (quadrant × group interaction: $F_{(2,98)} = 5.038$, $p < 0.01$; simple effects test: WMT + Sed vs WMT + Ex in TQ, $t_{24} = -2.694$, $p < 0.05$; different quadrants in WMT + Sed group, $F_{(2,51)} = 3.598$, $p < 0.05$) (**Figure 3E**).

To exclude the effect of swim speed on the performance in the water maze, we compared the swim speed during the recall test between the WMT groups with or without running exercise. No

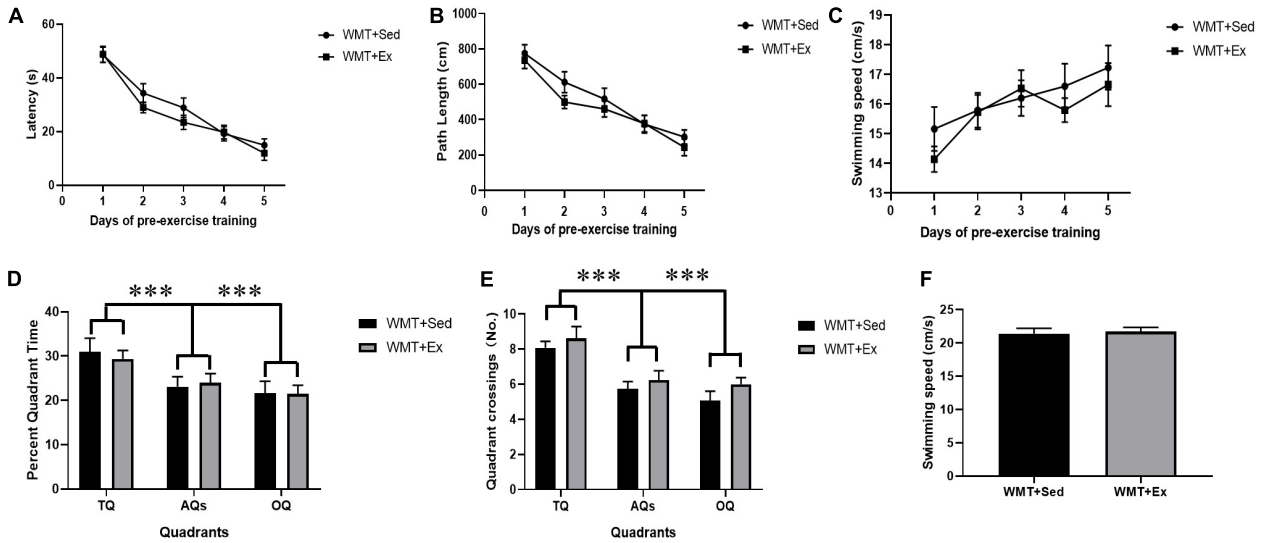

FIGURE 2 | Mice successfully acquired the spatial memory during pre-exercise learning. **(A,B)** Two-way repeated-measures ANOVA shows no difference between groups of mice that were sedentary with memory traces (WMT + Sed) and those with memory traces plus exercise (WMT + Ex) in their performance during pre-exercise training. **(C)** Comparison of swimming speed between the WMT + Sed and WMT + Ex groups during pre-exercise training. There is no difference in the swimming speed between groups. **(D,E)** Two-way ANOVA shows no difference between groups of mice in their performance during pre-exercise probe test, and mice shows significant larger time and more entries in the target quadrant (TQ) than that in the adjacent quadrants (AQs) and opposite quadrant (OQ). **(F)** Comparison of swimming speed between the WMT + Sed and WMT + Ex groups during probe test. There is no difference in the swimming speed between groups. Data are graphed as means ± SEM (n = 13) and analysis used two-way repeated ANOVA **(A–C)** and two-way ANOVA **(D,E)**. ***$p < 0.001$.

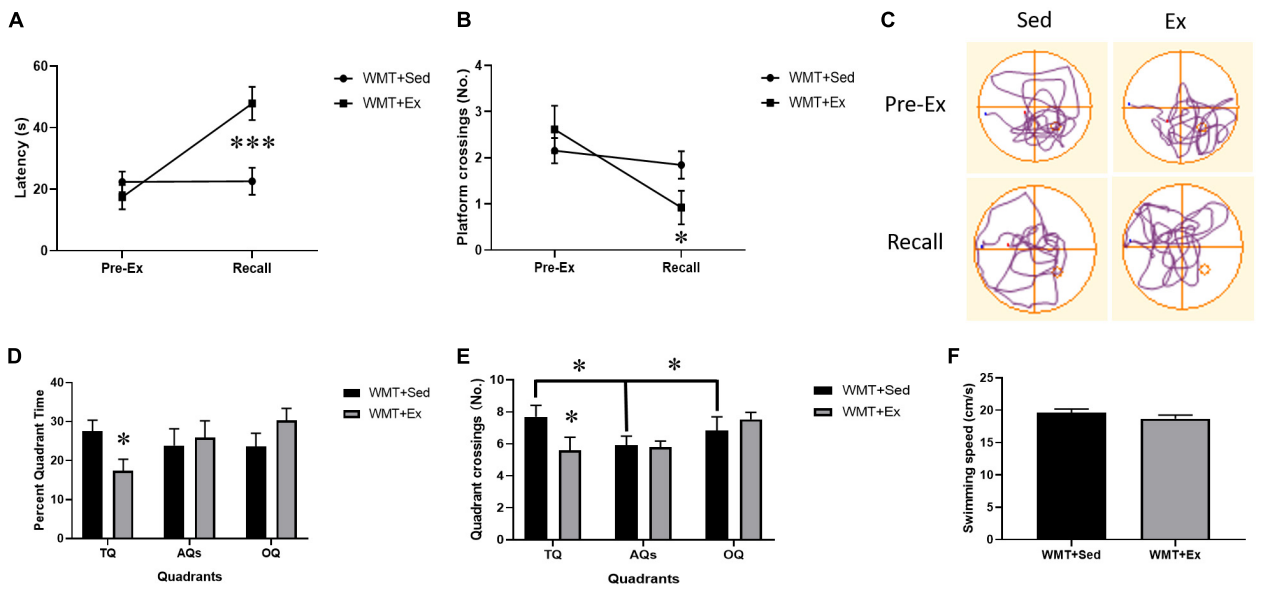

FIGURE 3 | Exercise facilitates the diminishment of acquired memory traces. **(A,B)** Comparison of memory retention (pre-exercise probe vs. recall) between the WMT + Sed and WMT + Ex groups after 4 weeks of housing in standard cages (Sed) or in cages equipped with a running wheel (Ex). There is a significant increase in the latency to find the platform location and decrease in the number of entries in the platform when compared with that during the pre-exercise probe test in the WMT + Ex group, whereas no significant change is detected for the WMT + Sed group. **(C)** Trajectories of mice exploring the maze during the pre-exercise probe and recall tests. **(D)** The percentage of time mice spend in different quadrants during the probe test of recall. Exercise significantly reduces mice exploration in the target quadrant (TQ) when comparing with sedentary group. No difference is found in the exploration in different quadrants (target: TQ, adjacent: AQs and opposite quadrants: OQ) for both groups. **(E)** The number of quadrant crossings during recall probe test. Mice in the WMT + Ex group show decreased the TQ crossings when comparing with mice in the WMT + Sed group. Mice in the WMT + Sed group exhibit more entries in the TQ than AQs and TQ. **(F)** Comparison of swimming speed between the WMT + Sed and WMT + Ex groups during probe test. There is no difference in the swimming speed between groups. Data are graphed as means ± SEM (n = 13) and were analyzed using two-way repeated ANOVA **(A,B)** and two-way ANOVA (D, E). *$p < 0.05$, ***$p < 0.001$.

difference was found between the WMT + Sed and WMT + Ex groups ($t_{24} = -1.124$, $p > 0.05$) (**Figure 3F**).

Experience Equally Facilitates Associative Learning Despite Significantly Lower Memory Retention in Exercise Group

To investigate whether spatial memory traces decreased by exercise have any effects on new spatial learning, we compared the latencies and path lengths to reach the target platform during the 5 days of post-exercise training among the groups using three-way repeated ANOVA. The results indicated that exercise (Ex) and experience (WMT) both had significant facilitation on new spatial learning with decreased target platform latencies (exercise main effect: $F_{(1,38)} = 21.389$, $p < 0.01$; experience main effect: $F_{(1,38)} = 32.947$, $p < 0.01$; exercise × experience interaction: $F_{(1,38)} = 1.785$, $p > 0.05$) and reduced path lengths (exercise main effect: $F_{(1,38)} = 10.036$, $p < 0.01$; experience main effect: $F_{(1,38)} = 32.246$, $p < 0.001$; exercise × experience interaction: $F_{(1,38)} = 1.785$, $p > 0.05$) during 5 days' reversal learning (**Figures 4A,B**). Swimming speeds were also examined during reversal learning using three-way repeated-measures ANOVA. The results showed that the swimming speed in the NMT groups increased during the 5 days of learning, whereas there were no significant changes in the WMT groups (day × experience interaction: $F_{(4,152)} = 3.898$, $p < 0.01$; simple effects test: in the NMT group, day main effect, $F_{(4,96)} = 3.534$, $p < 0.05$; in the WMT groups, day main effect, $F_{(4,96)} = 1.793$, $p > 0.05$). In addition, exercise increased the swimming speed of mice only in the NMT groups (exercise × experience interaction: $F_{(1,38)} = 8.916$, $p < 0.01$; simple effects test: in the NMT group, exercise main effect, $F_{(1,14)} = 6.285$, $p < 0.05$; in the WMT groups, exercise main effect, $F_{(1,24)} = 2.546$, $p > 0.05$) (**Figure 4C**).

During the reversal probe test, we compared the escape latency and platform entries among the different groups using two-way ANOVA. Exercise and experience both showed facilitation of searching for the platform location with decreased latency (exercise main effect: $F_{(1,38)} = 4.554$, $p < 0.05$; experience main effect: $F_{(1,38)} = 4.583$, $p < 0.05$; exercise × experience interaction: $F_{(1,38)} = 1.112$, $p > 0.05$) and increased number of platform entries (exercise main effect: $F_{(1,38)} = 6.463$, $p < 0.05$; experience main effect: $F_{(1,38)} = 10.112$, $p < 0.01$; exercise × experience interaction: $F_{(1,38)} = 0$, $p > 0.05$) (**Figures 5A,B**). The swimming speeds among the groups during the probe test were examined using two-way ANOVA. Exercise increased the swimming speed only in the NMT group (exercise × experience interaction: $F_{(1,38)} = 9.216$, $p < 0.01$; simple effects test: NMT + Sed vs. NMT + Ex, $t_{14} = 4.734$, $p < 0.001$; WMT + Sed vs. WMT + Ex, $t_{24} = -0.259$, $p > 0.05$) (**Figure 5C**). In addition, we investigated the percentage of time spent in the various quadrants among the groups. The results showed that exercise and experience both increased the time mice spent in the TQ during the 60 s exploration of the probe test (exercise main effect: $F_{(1,38)} = 10.239$, $p < 0.01$; experience main effect: $F_{(1,38)} = 6.817$, $p < 0.05$; exercise × experience interaction: $F_{(1,38)} = 2.913$, $p > 0.05$) and reduced the percent quadrant time in the OQ (exercise main effect: $F_{(1,38)} = 5.128$, $p < 0.05$; experience main effect: $F_{(1,38)} = 5.257$, $p < 0.05$; exercise × experience interaction: $F_{(1,38)} = 1.765$, $p > 0.05$). No difference was found in the time spent in the AQs for mice among the different groups (**Figure 5D**). The quadrant crossings were also examined, and the results showed that exercise and experience both increased the TQ crossings (exercise main effect: $F_{(1,38)} = 14.524$, $p < 0.001$; experience main effect: $F_{(1,38)} = 10.028$, $p < 0.01$; exercise × experience interaction: $F_{(1,38)} = 0.014$, $p > 0.05$). When analyzing the OQ crossings, we found that exercise increased the OQ crossings in the NMT groups, but decreased the OQ crossings in the WMT groups (exercise main effect: $F_{(1,38)} = 0.348$, $p > 0.05$; experience main effect: $F_{(1,38)} = 0.037$, $p > 0.05$; exercise × experience interaction: $F_{(1,38)} = 13.385$, $p = 0.001$; simple effects test: NMT + Sed vs. NMT + Ex, $t_{24} = 2.576$, $p < 0.05$, WMT + Sed vs. WMT + Ex, $t_{24} = -3.079$, $p < 0.01$). For the AQ crossings, exercise showed an enhanced effect on the crossing numbers in the AQs for both the NMT and WMT groups (exercise main effect: $F_{(1,80)} = 5.861$, $p < 0.05$; experience main effect: $F_{(1,80)} = 0.281$, $p > 0.05$; exercise × experience interaction: $F_{(1,80)} = 3.935$, $p > 0.05$) (**Figures 5E,F**).

Exercise Facilitates the Development of a Direct Search Strategy in the Water Maze

To investigate the search strategies in the different groups during post-exercise learning, we compared the Wishaw's index (the percent time in the corridor connecting the start position with the platform) using two-way ANOVA. The results showed that mice in both NMT + Ex and WMT + Ex groups preferred the direct search strategy with more percent quadrant time in the corridor [exercise main effect: $F_{(1,38)} = 6.551$, $p < 0.05$; experience main effect: $F_{(1,38)} = 0.09$, $p > 0.05$; exercise × experience interaction: $F_{(1,38)} = 0.4$, $p > 0.05$] (**Figures 6A,B**).

Memory Traces Differentially Affect New Spatial Learning in Sedentary and Exercise Groups

We investigated how former memory traces affect associated new learning. To this end, we first compared the number of crossings over the former platform location (after the platform was moved to a new location) between the WMT + Sed and WMT + Ex groups during 5 days of reversal learning using a two-way repeated-measures ANOVA. The results showed that both groups decreased the number of crossings over the former platform location with reversal learning. However, the number of crossings was significantly lower in the WMT + Ex group than in the WMT + Sed group throughout the learning process (day main effect: $F_{(4,96)} = 3.616$, $p < 0.01$; group main effect: $F_{(1,24)} = 9.548$, $p < 0.01$; day × group interaction: $F_{(4,96)} = 0.25$, $p > 0.05$) (**Figure 7A**).

We then compared the WMT + Sed and WMT + Ex groups for the percentage of time spent in the opposite quadrant where the platform had been originally located, that is, the

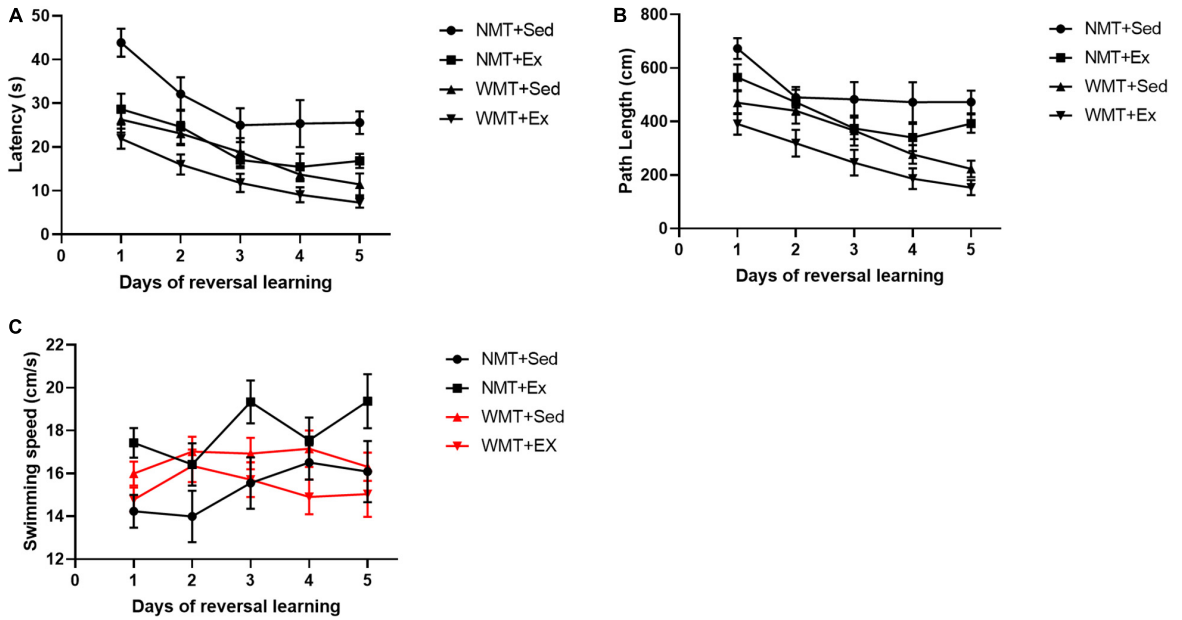

FIGURE 4 | Weakened memory traces induced by exercise still facilitate new learning. **(A,B)** Prior experience in the Morris water maze facilitates later reversal learning in the maze in both the sedentary (Sed) and exercise (Ex) groups during 5 days acquisition phase. And no matter mice in with no memory trace (NMT) or with memory trace (WMT) groups, mice engaged in exercise training performed much better than that in sedentary during new learning. **(C)** Swimming speed during reversal learning. Swimming speed increases during the 5 days of learning in NMT groups. Exercise increases mice swimming speed in the NMT groups. Data analysis used three-way repeated ANOVA.

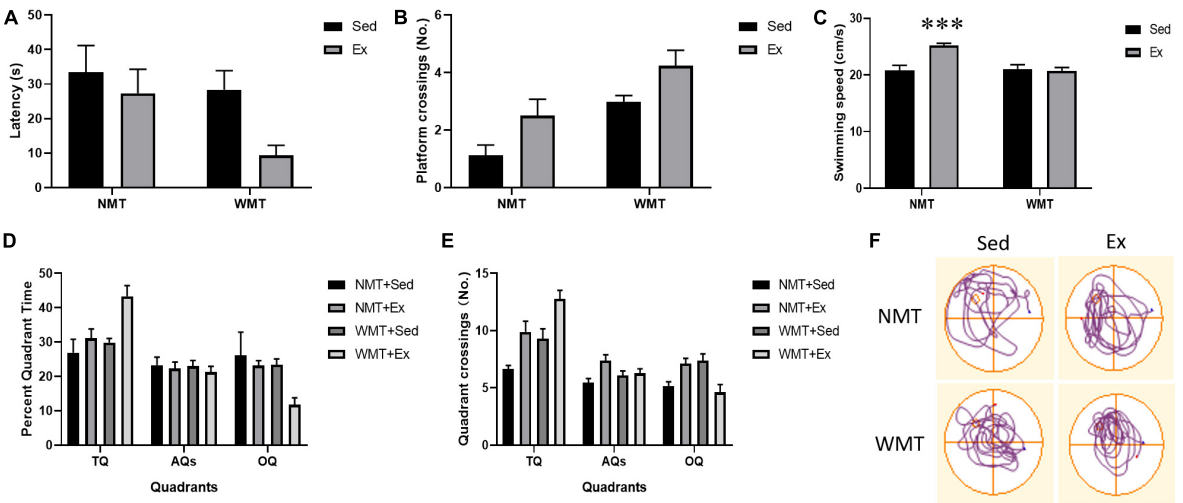

FIGURE 5 | Weakened memory traces induced by exercise facilitate consolidation of new memory. **(A,B)** Experience and exercise both facilitate decreasing target platform latency and enhance the number of crossings in the platform location during the final probe test. **(C)** Swimming speed during the final probe test. Exercise increased mice swimming speed in the NMT groups. **(D,E)** Experience and exercise increase the percent quadrant time and number of crossings in the target quadrant (TQ). Similarly, both experience and exercise decreased the percent quadrant time in the opposite quadrant (OQ). Exercise increases the number of OQ crossings in the NMT groups, whereas it decreases the number of OQ crossings in the WMT groups. Exercise also increases adjacent quadrants (AQs) crossings only in the NMT groups, with no effect on the percent quadrant time in AQs in both the NMT and WMT groups. **(F)** Trajectories of mice exploring the maze during the final probe test. Data are graphed as means ± SEM (n = 8–13); Data analysis used two-way ANOVA. $^{***}p < 0.001$.

OQ, and the new platform location, that is, the new target quadrant (TQ). We found that exercise facilitated acquisition of the new TQ location during the 5 days of reversal training in the WMT + Ex group, with increased time in the TQ (group main effect: $F_{(1,24)} = 44.337$, $p < 0.001$; day × group interaction: $F_{(4,96)} = 1.794$, $p > 0.05$, two-way repeated-measures ANOVA) and decreased time in the OQ (group main effect: $F_{(1,24)} = 21.304$, $p < 0.001$; day × group interaction:

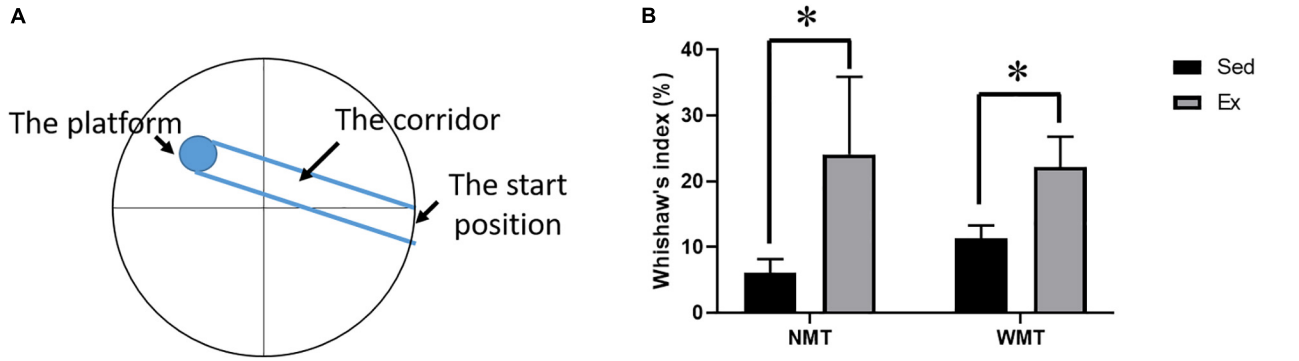

FIGURE 6 | Search strategies in different groups. **(A)** Scheme of maze configuration showing the corridor. **(B)** Different search strategies among groups. Mice in exercise (Ex) groups, including NMT + Ex and WMT + Ex groups, prefer a direct search strategy with more percent quadrant time in the corridor (connecting the start position with the platform) before the first entry to the platform. Data are graphed as means ± SEM (n = 8–13); Data analysis used two-way ANOVA. *$p < 0.05$.

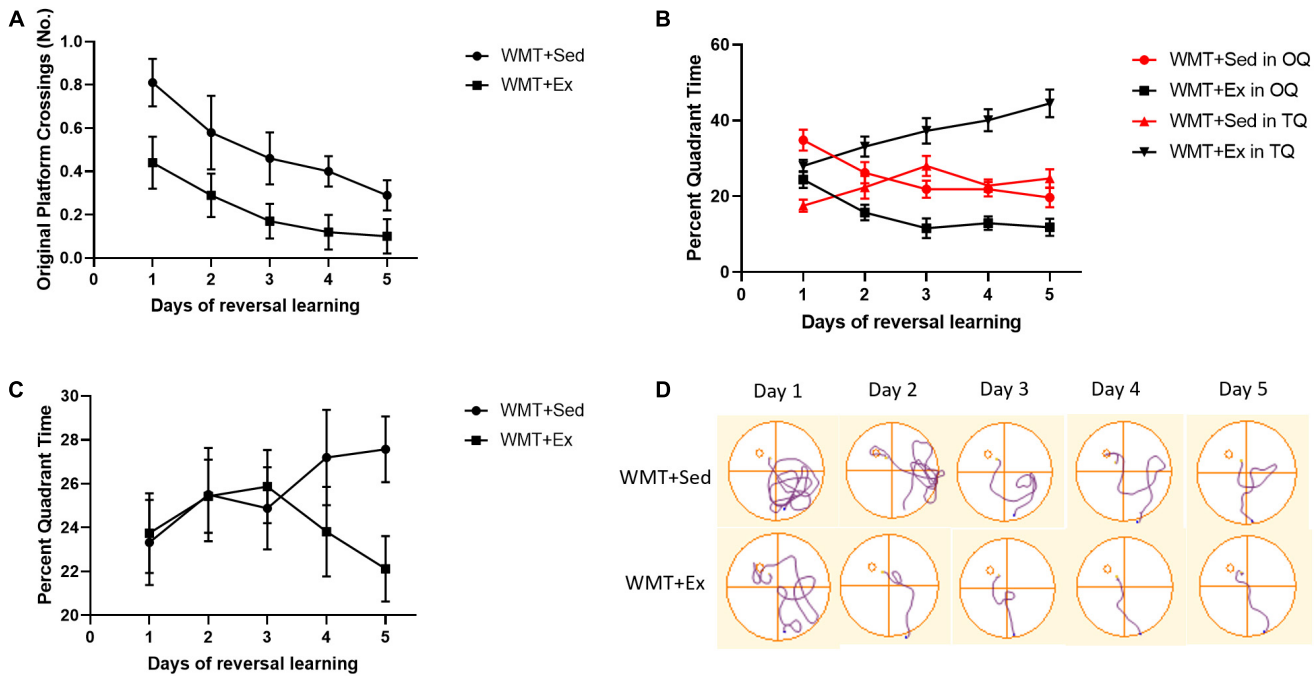

FIGURE 7 | Lower learning efficiency in sedentary mice may be attributable to maintained memory traces. **(A)** Comparison of the number of crossings over the original platform (the platform during pre-exercise training) between groups of mice that were sedentary with memory traces (WMT + Sed) and mice with memory traces that exercised (WMT + Ex). Mice in the WMT + Sed group show significantly higher numbers of crossings over the original platform location during 5 days of reversal learning than mice in the WMT + Ex group. **(B)** Comparison of the percentage of time spent in the new target quadrant (TQ) and opposite quadrant (OQ) between the WMT + Sed and WMT + Ex groups during reversal learning. Both groups increase exploration time in the TQ and decrease exploration time in the OQ. However, the difference in exploration time between the TQ and the OQ in the WMT + Sed group is statistically significant only on the first day of reversal learning. By contrast, mice in the WMT + Ex group spend significantly more time in the TQ and less time in the OQ during the last 4 days of reversal learning, but not on the first day. **(C)** Comparison of the percentage of time spent in the adjacent quadrants (AQs) between the WMT + Sed and WMT + Ex groups during reversal learning. Both groups spent similar time in AQs during 5 days of learning. **(D)** The trajectories of mice exploring the maze during the 5 days of reversal learning. Data are graphed as means ± SEM (n = 13) and were analyzed using two-way repeated-measures ANOVAs.

$F_{(4,96)} = 0.161$, $p > 0.05$, two-way repeated-measures ANOVA). However, when we compared the difference in the time spent in the new TQ and OQ between the WMT + Sed and WMT + Ex groups, we discovered that the two groups were using different strategies. During reversal training, the percentage of time spent in the new TQ increased and the percentage of time spent in the OQ decreased for mice in the WMT + Sed group (quadrant × day interaction: $F_{(4,96)} = 9.728$, $p < 0.01$, two-way repeated-measures ANOVA); but the percentage of time spent in the TQ was significantly different (lower) from that spent in the OQ only on the first day of training ($t_{24} = 5.452$, $p < 0.01$). By contrast, mice in the WMT + Ex group spent

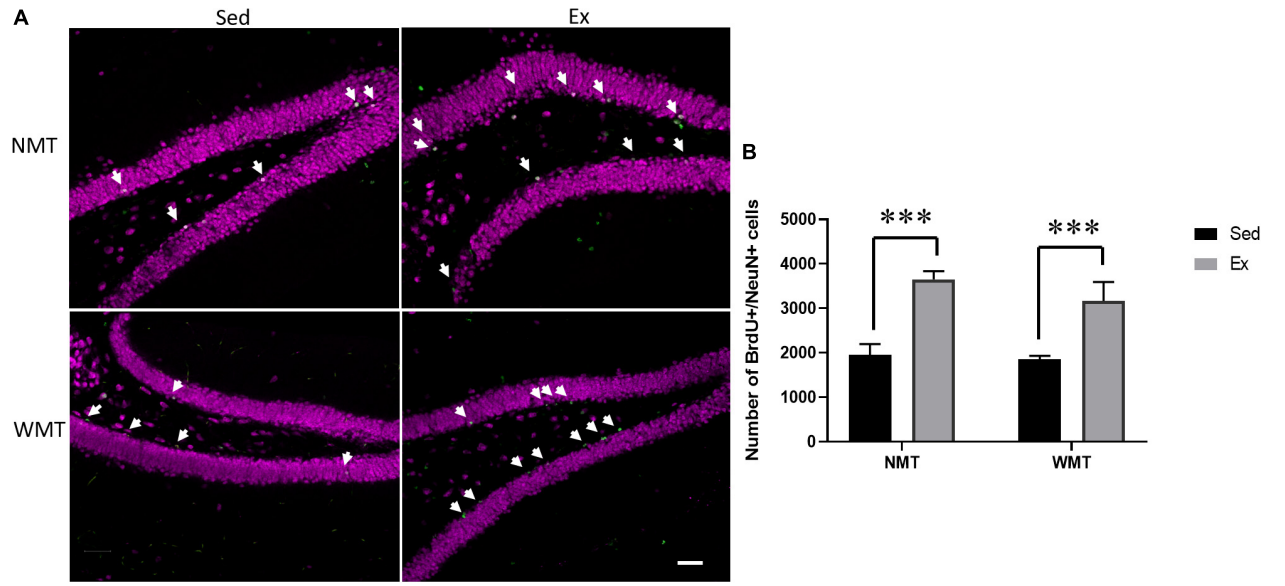

FIGURE 8 | Exercise facilitates adult-born neurons. **(A)** Representative images of adult-born neurons labeled with 5-bromo-2′-deoxyuridine (BrdU; indicated with arrows) and NeuN in the hippocampal dentate gyrus (DG) of mice in the sedentary (Sed) and exercise (Ex) groups. NMT represents no memory traces; WMT, with memory traces; scale bar, 50 μm. **(B)** Four weeks of wheel running enhances neurogenesis in the DG. Data are graphed as means ± SEM (n = 3–4); Data analysis used two-way ANOVA. ***$p < 0.001$.

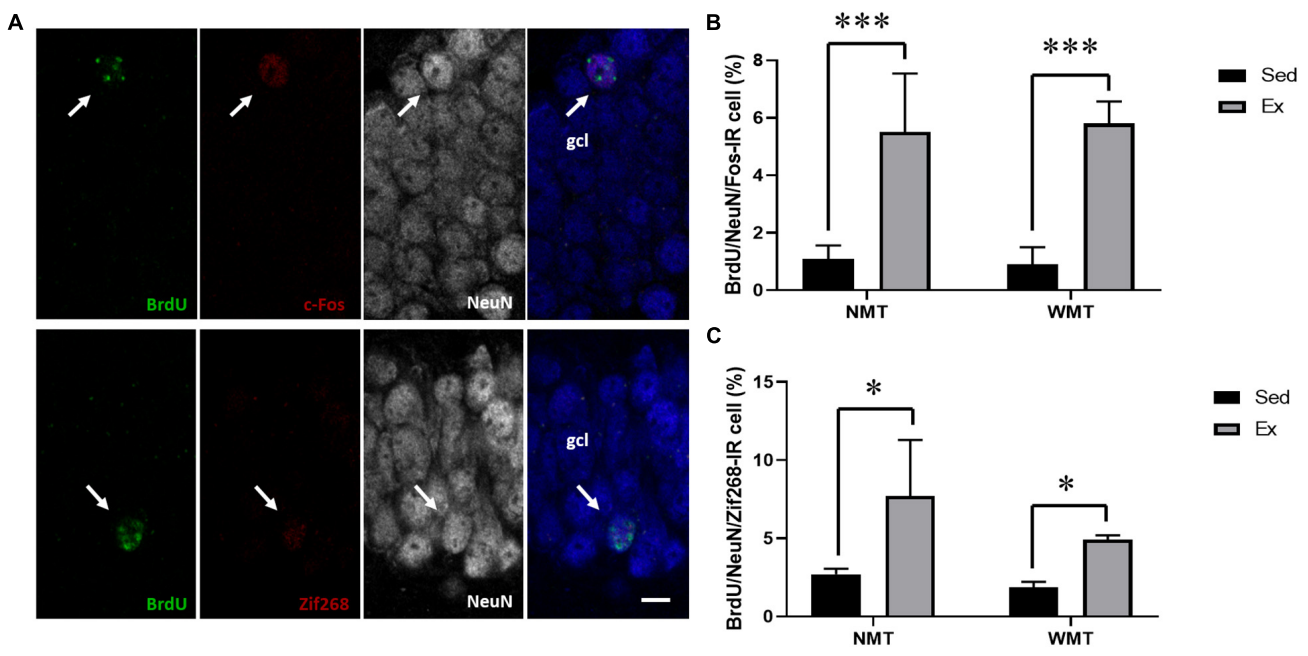

FIGURE 9 | Spatial memory induces activation of IEGs in adult-born neurons. **(A)** Representative images of adult-born neurons activated during the probe test of reversal learning. The fourth column is the merged image. Arrows indicate the respective labeling; scale bar, 10 μm. **(B,C)** Percentage of adult-born neurons expressing c-Fos or Zif268 in the granular cell layer (gcl) of the hippocampal dentate gyrus. Ex indicates exercise; NMT, mice with no memory traces; WMT, mice with memory traces; and Sed, sedentary. Data are graphed as means ± SEM (n = 3–4). Data analysis used two-way ANOVA. *$p < 0.05$, ***$p < 0.001$.

the same percentage of time in the two quadrants on the first day of reversal training, but time spent in the TQ increased with training days while time spent in the OQ decreased with training (quadrant main effect: $F_{(1,24)} = 105.67$, $p < 0.001$; quadrant × day interaction: $F_{(4,96)} = 11.244$, $p < 0.001$, two-way repeated-measures ANOVA) (**Figure 7B**).

To confirm that the lower learning efficiency for mice in WMT + Sed groups was only due to the longer exploration in

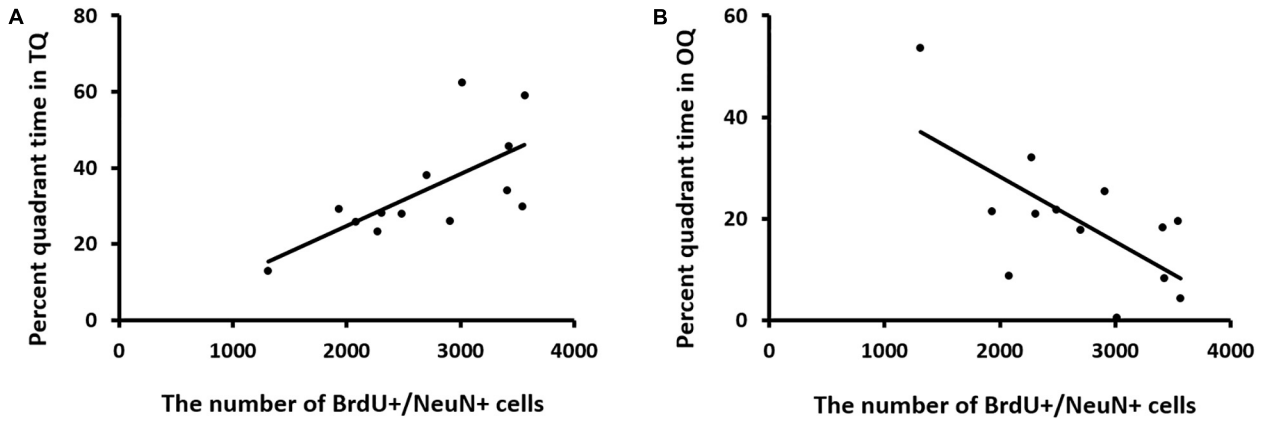

FIGURE 10 | Correlation between neurogenesis and memory retention in reversal learning. **(A,B)** Neurogenesis is positively related to the percentage of time spent in the target quadrant (TQ), while it is negatively related to the time spent in the opposite quadrant (OQ). Data were analyzed using Pearson correlation analysis.

OQ, we also compared the exploration time in AQs between the WMT + Sed and WMT + Ex groups during 5 days of reversal learning. The results showed that WMT + Sed and WMT + Ex groups spent similar time in the AQs during the 5 days of reversal learning (group main effect: $F_{(1,50)} = 2.003$, $p > 0.05$; day × group interaction: $F_{(4,200)} = 1.092$, $p > 0.05$, two-way repeated-measures ANOVA) (**Figures 7C,D**).

Exercise Enhances Hippocampal Neurogenesis

The number of adult-born neurons labeled with BrdU/NeuN was compared among the groups using a 2 (experience: NMT, WMT) × 2 (group: Sed, Ex) two-way ANOVA. The results showed that there was a significant main effect of group between the exercise and sedentary groups ($F_{(1,11)} = 29.207$, $p < 0.001$) (**Figure 8**).

Exercise Enhances the Activation of Adult-Born Neurons in New Associative Learning

immediate early genes, such as c-Fos and Zif268 (also known as early growth response 1 or Egr1), are commonly used to investigate the activation of neurons during learning and other stimulation. Here, we explored the participation of the adult-born neurons in new spatial learning under different conditions by analyzing the proportion of newborn neurons labeled with BrdU/NeuN/c-Fos or BrdU/NeuN/Zif268 in the hippocampal DG. The results showed that the proportion of adult-born neurons expressing c-Fos in the exercise groups was significantly higher than that in the sedentary groups, $F_{(1,10)} = 21.254$, $p = 0.001$. Similarly, the proportion of adult-neurons expressing Zif268 in the exercise groups was also significantly higher than those in the sedentary groups, $F_{(1,10)} = 6.985$, $p < 0.05$. However, no significant difference was found in the c-Fos or Zif268 expression between the NMT and WMT groups (**Figure 9**).

We also analyzed the relationship between neurogenesis and behavioral performance (percent quadrant time in the TQ and OQ during the reversal probe test) using Pearson correlation analysis. A positive correlation was found between neurogenesis and the time mice spent in the TQ (Pearson correlation coefficient, $r = 0.676$; $n = 13$), whereas a negative correlation was found between neurogenesis and the time mice spent in the OQ (Pearson correlation coefficient, $r = -0.662$; $n = 13$) (**Figure 10**).

DISCUSSION

Previous studies have shown that exercise facilitates forgetting but enhances reversal learning by decreasing PI (Epp et al., 2016). PI, a type of memory interference, usually occurs when consolidated memories inhibit current learning. However, the effect of old memories on new learning is not only negative, it can also be positive, such as in proactive facilitation, which refers to old memories enhancing new learning when the content of the new and old memories overlaps. In the present study, we did not find PI but found proactive facilitation effects of previous memory traces on spatial learning in both the exercise and sedentary groups. Although there was a decrease in memory retention in the exercise group, the positive impact of the previous experience on the subsequent new learning was preserved. An indirect search strategy might have survived from the previous experience to facilitate new learning. In addition, exercise enhanced the learning performance in both the NMT and WMT groups. This facilitation effect might result from a higher activation of adult-born neurons.

It is widely accepted that exercise facilitates learning and memory. However, most of the studies showing this facilitation have focused on the effects of exercise on the performance of learning tasks following the exercise, whereas few studies have investigated the effects of exercise on the memories consolidated before the exercise. Studies on neurogenesis-induced forgetting have indicated that exercise facilitates the forgetting of previous memory traces through neurogenesis (Akers et al., 2014; Gheorghe et al., 2018). Epp et al. (2016) further suggested that neurogenesis-induced forgetting might

reduce PI from remote memory traces when learning items with overlapping information (Epp et al., 2016). Our results were consistent with these previous studies, showing that exercise enhanced the forgetting of remote memories. In our study, we found an increased latency to find the platform and a decreased number of crossings in the target platform location after 4 weeks' delay in the WMT + Ex group, whereas no significant changes were found in the WMT + Sed group. More crossings in the TQ were also found in the WMT + Sed group when compared with those in the WMT + Ex group. In addition, the number of crossings in the TQ were significantly higher than that in the other quadrants in the WMT + Sed group. These data indicated that, to some extent, there was a decrease in memory retention for mice in the WMT + Ex group, while the previous memory traces were retained for mice in the WMT + Sed group. Conflicting data were also found when examining memory retention in the WMT + Sed group, which showed no difference in the percent quadrant time among the different quadrants during the recall test. The non-preferred exploration in the TQ for mice in the WMT + Sed group might have been because the probe test during recall was the second probe test without a platform in the pool. Thus, although these animals remembered the position of the platform and explored that location immediately after entering the pool, when they did not find the platform where they expected it to be, they might have been unsure whether it was still in that quadrant and therefore explored the other quadrants as well. In addition, we did not find that exercise decreased PI when we investigated the effects of exercise-induced forgetting on new learning in a similar paradigm. Indeed, no PI from previous memory traces was found when mice underwent new learning during the reversal training. In the present study, we conducted post-exercise training of mice with or without remote memory traces. When we compared these groups after the spatial learning task, we found that the experience of the mice that had been exposed to the MWM task facilitated the associative learning in the reversal spatial task. This facilitation was observed for experienced mice in both the exercise and sedentary groups. It should be pointed out that although significantly lower memory retention was found in the WMT + Ex group, the experience of pre-exercise training equally facilitated the acquisition of the new platform location during reversal learning. These results indicated that the retention of the platform location might be unnecessary for the subsequent associated learning. The strategy learned from experience might be the key factor to benefit new learning. Previous studies have shown the facilitation of proactive experience on later associative learning for both motor and episodic memory tasks (Verneau et al., 2015; Zimmermann et al., 2016). This kind of positive transfer makes acquiring information more efficient. We suggest that the strategies obtained from experience survived exercise-induced forgetting. These strategies remained to benefit the subsequent new learning. To understand the search strategies during reversal learning, we examined Whishaw's index during the reversal probe test. The data showed that mice in the exercise groups spent more time in the corridor connecting the start position with the platform before the first entry in the platform location, and there was no difference between the NMT + Ex and WNT + Ex groups. This indicated that exercise facilitated the development of a direct search strategy during water maze learning. However, the surviving strategies from the previous experience might be indirect search strategies. The type of search strategies used will require further study. Another study using paired-associate learning to investigate the benefit of neurogenesis-induced forgetting on learning of conflicting information found that the neurogenesis-induced forgetting decreased PI in new learning with content that was highly conflicting, but no effect was found for learning of information with low conflict interference (Epp et al., 2016). In our study, we utilized a classic spatial learning paradigm, the MWM, to explore the effects of exercise-induced forgetting on new learning (reversal learning) using the same water maze. We found enhanced facilitation but not reduced interference from the remote spatial learning memory. The inconsistency between the results of the previous study and ours might be because of the different levels of difficulty of these two tasks. A study by Epp et al. (2016) exposed mice to two contexts (e.g., context A: white container; context B: striated container) with one of two scented beddings (e.g., odor 1: coffee; odor 2: cinnamon). Mice responded to specific context-odor pairs to gain rewards (e.g., rewarded for responding to odor 1 in context A and responding to odor 2 in context B). During reversal learning with high interference, the rewarded pairs were reversed (i.e., rewarded for responding to odor 2 in context A and to odor 1 in context B). Based on the hippocampal cognitive map theory, each context corresponds to a specific hippocampal map (environmental cues influence behavior), and "remapping" occurs when the context changes (Kubie et al., 2019). Therefore, there are two maps in the paired-associate task and two "remapping" processes during reversal learning. However, in the present study, there was only one hippocampal cognitive map during the acquisition of the MWM task, and one "remapping" process during reversal learning in the MWM. As a result, during new learning of additional conflicting information in the paired-associate task, memory traces may have provided more interference than facilitation. In addition, as previous studies have shown, swimming-induced stress might have a negative effect on cognitive performance (Shen et al., 2019). Thus, the poorer performance of mice in the NMT group during the acquisition of post-exercise spatial learning might, to some extent, result from swimming-induced stress. Exercise-induced enhancement in swimming speed was found in the NMT groups. Therefore, whether the decreased latency in the NMT + Ex group was affected by the increased swimming speed is unclear. Despite this, we can assert the beneficial effect of exercise and experience on new learning through the results of the path length, which is not affected by swimming speed. In addition, we found that exercise increased exploration in the TQ during the probe test in the NMT groups. These results are consistent with previous studies that have shown the beneficial effects of exercise on memory retention (Park et al., 2018; Leem et al., 2019).

Computer model results have suggested that the integration of adult-born neurons into a neural circuit results in neurogenesis-induced forgetting (Weisz and Argibay, 2012; Aimone, 2016).

New adult-born neurons integrating into a synaptic circuit might either replace or coexist with the existing synapse, which can lead to forgetting of consolidated memories (Finnegan and Becker, 2015). In addition, neurons that are newly born at certain stages (about 3–4 weeks in mice) have special characteristics that lead them to be more sensitive than mature neurons during memory encoding. Researchers have reported that immature neurons of mice between 1 and 1.5 months of age express more *N*-methyl D-aspartate receptor subtype 2B (NR2B) receptors, which play a main role in neuroplasticity (Ge et al., 2007). Furthermore, less sensitivity to inhibitory interneurons has been found in adult-born neurons (Malleret et al., 2010). Therefore, neurogenesis is considered to be the main neuroplasticity factor mediating the enhancement of cognitive function after exercise. In the present study, we investigated the participation of adult-born neurons in post-exercise learning by labeling cells with antibodies to BrdU, NeuN, and IEGs. As shown in several previous studies, increased neurogenesis was found in the hippocampus of mice in the exercise groups. In addition, we found a greater proportion of newborn neurons positive for c-Fos and Zif268 in the exercise groups than in the sedentary groups, indicating increased activation of these newborn neurons when obtaining a spatial memory in mice that exercised. A previous study has shown that early-age exercise enhances the activity of adult-born neurons in the hippocampal DG after learning (Shevtsova et al., 2017). Other researchers also find that running exercise facilitates memory encoding by reorganizing the adult-born neurons (Vivar et al., 2016). However, there are conflicting results showing that neurogenesis has no effects on spatial learning in rats (Groves et al., 2013). Controversial findings also appear in studies examining the effect of neurogenesis on memory retention. Numerous studies in mice have shown the facilitation of neurogenesis on forgetting by using spatial memory or fear conditioning tasks (Akers et al., 2014; Epp et al., 2016), whereas Kodali et al. (2016) found no effect of neurogenesis on forgetting. Such results might suggest differential effects of neurogenesis on various types of cognition (e.g., spatial learning) among different species. In our study, we investigated the association between neurogenesis and memory retention during reversal learning. We found a positive correlation between neurogenesis and the time mice spent in the TQ and a negative correlation between neurogenesis and the time mice spent in the OQ during the reversal probe test. However, because correlation does not necessarily imply causation, it is unclear whether neurogenesis contributed to the changes in memory retention. In addition, our results showed that the activity of the newborn neurons was not different between groups with or without previous memory traces, which might suggest that adult-born neurons might equally participate in memory encoding whether in a similar situation or a totally novel context. Considering the results of a study by Bostock et al. (1991), a given representation in the hippocampus reliably reactivates when animals return to the corresponding environment. Pignatelli et al. (2019) suggested that successful recall of a contextual memory is influenced by the activation of the same engram cells activated during memory encoding. Such evidence provides a basis for the assertion that a similar group of engram cells would be activated when animals return to the same environment. Therefore, neurons activated during the probe test would involve the same group of engram cells activated during learning in water maze tasks.

During reversal learning for the WMT groups, we found a lingering exploration of the former TQ in the sedentary group, which might explain the lower learning efficiency in the sedentary group when compared with the exercise group. During reversal learning, hippocampal remapping occurs, and another set of place cells is activated (Kubie et al., 2019). Adult-born neurons are more sensitive than mature neurons in responding to stimulation (Beining et al., 2017). This might lead to the preferred involvement of the adult-born neurons in hippocampal remapping. The participation of newborn neurons in hippocampal remapping benefits pattern separation, a process distinguishing overlapping contextual representations. Thus, mice in the exercise group, which had more adult-born neurons, may have more easily distinguished the changed context during reversal learning and more rapidly achieved new memories.

In summary, our study explored the proactive facilitation effect of diminished spatial memory induced by exercise on the acquisition of a subsequent related memory. We found that although the previous memory traces were weakened after 4 weeks of exercise, the previous experience still increased the efficiency of new learning in a similar paradigm. Further investigation of the search strategies involved in spatial learning indicated that previous experience might have provided indirect search strategies for subsequent related new learning, and these search strategies were not affected by exercise intervention. Exercise facilitated learning efficiency through the acquisition of a direct search strategy. We also investigated the participation of newborn neurons in new learning between animals with or without a similar experience and found that the activation was equal. However, to what extent these newborn neurons participated in this process and whether the existing mature neurons participated in this process will require further study.

DATA AVAILABILITY STATEMENT

The datasets are available on request to any qualified researcher. The raw data supporting the conclusions of this article will be made available by the authors.

ETHICS STATEMENT

All animal experiments were ethically reviewed, approved, and conducted according to the animal care guidelines of the Ethics Committee of Shanghai University of Sport.

AUTHOR CONTRIBUTIONS

CZ and RL were responsible for the study concept and design and provided critical revision of the manuscript for important intellectual content. CL contributed to the research conduction and drafted the manuscript. All authors critically reviewed the content and approved the final version for publication.

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The 24-Form Tai Chi Improves Anxiety and Depression and Upregulates miR-17-92 in Coronary Heart Disease Patients After Percutaneous Coronary Intervention

Jia Liu[1†], Ping Yu[1†], Wei Lv[2] and Xinxin Wang[2*]*

[1] Department of Cardiovascular Medicine, The First Hospital of Jilin University, Changchun, China, [2] Department of Cadre Ward, The First Hospital of Jilin University, Changchun, China

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Edited by:
Frederico C. Pereira, University of Coimbra, Portugal

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Filiz Tuna, Trakya University, Turkey

***Correspondence:**
Wei Lv
lvweijlu@126.com
Xinxin Wang
wangxinxin1949@126.com

[†] These authors have contributed equally to this work

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Background: Anxiety and depression are common symptoms in patients with coronary heart disease (CHD) after percutaneous coronary intervention (PCI). The 24-form Tai Chi may exert a protective function for CHD patients after PCI by improving anxiety and depression.

Methods: Patients who received PCI after 1–4 days were randomly assigned to the 24-form Tai Chi group (TG) and the control group (CG). The differences in anxiety and depression, using the Medical Outcomes Study 36–item Short–Form Health Survey (SF-36), before and after an average of 10 months of Tai Chi intervention were compared in both groups to analyze the effects of Tai Chi on the emotion and the life quality of CHD patients. Meanwhile, the relative levels of miR-17-92 were measured by using real-time qPCR. The association between the relative levels of miR-17-92 and the anxiety and the depression of CHD patients after PCI was analyzed. Adjusted Cox models were used to explore the effect of Tai Chi exercise in CHD patients.

Results: After 10 months of intervention, the changes in the anxiety subscale ($P = 0.002$), in the depression subscale ($P = 0.008$), and in the stress ($P = 0.015$) scores were higher in the TG group when compared to those of the CG group. The proportion of anxious ($P = 0.045$) and depressed subjects ($P = 0.042$) in the TG group was lower than that in the CG group. On the other hand, the increase in the SF-36 scores and in the relative levels of miR-17-92 was significantly higher in the TG group when compared with that of the CG group ($P < 0.05$). The serum level of miR-17-92 had a negative correlation with the anxiety, the depression, and the stress scores ($P < 0.01$).

Conclusion: The 24-form Tai Chi improved the anxiety and the depression symptoms and upregulated the miR-17-92 levels in CHD patients after PCI.

Keywords: 24-form Tai Chi, anxiety, depression, SF-36, coronary heart disease, percutaneous coronary intervention, miR-17-92

Abbreviations: BP, bodily pain; CHD, coronary heart disease; GH, general health perceptions; HADS, Hospital Anxiety and Depression Scale; MACEs, major adverse cardiovascular events; MH, mental health; PCI, percutaneous coronary intervention; PF, physical functioning; PSS, Perceived Stress Scale; RE, role limitations due to emotional health; RP, role limitation due to physical health; SF, social functioning; SSA, subsyndromal anxiety; SSD, subsyndromal depression; VT, vitality.

INTRODUCTION

Coronary heart disease is a major disease that threatens human life and health and is one of the leading causes of death (Grabovac et al., 2018; Zhao et al., 2019). PCI is widely used in the treatment of CHD (Cheng et al., 2019; Dayoub et al., 2019). Although the cases of the patients with coronary artery restenosis and thrombosis can be reduced from 30–40 to 15% after 1 year of PCI, restenosis, and thrombosis are still serious clinical problems (Jaffery et al., 2011; Hofma et al., 2015). Therefore, controlling the risk factors of CHD is still necessary to prevent the occurrence of MACEs.

Depression (Hennessy et al., 2018; Monk et al., 2018; Zotcheva et al., 2019) and anxiety (Kasten et al., 2019; Ullmann et al., 2019; Zotcheva et al., 2019) are known indicators of poor outcomes in CHD (Palacios et al., 2018). Heart attack and PCI surgery also result in anxiety and depression (Zhang, 2015b). Anxiety and depression can produce multiple negative effects on patients by reducing their compliance with the treatment (Gehi et al., 2005; Bauer et al., 2012). Furthermore, the anxiety and the depression of CHD patients often occur together after PCI (Kala et al., 2016). There is still no effective way to control the symptoms of anxiety and depression.

Tai chi is a popular mind–body exercise in China that combines Chinese martial arts and meditative movements, and it can promote body balance (Adler et al., 2019), lessen depression, and improve cognitive function (Zhang et al., 2014; Sungkarat et al., 2018). The exercise needs mental concentration, physical balance, and muscle relaxation and shows a great potential in rehabilitating medical and psychological status (Yeh et al., 2009). Tai Chi even effectively treats adolescents with depression (Zhang et al., 2018). SSD and SSA are common in the elderly population and can cause suicide risk and disability. Tai Chi has been proven to improve SSD and SSA symptoms over 1 year of intervention (Rawtaer et al., 2015). Meta-analysis has shown that Tai Chi intervention reduced the Hamilton Depression Scale and Hamilton Anxiety Scale scores of stroke patients (Yang et al., 2018). Another meta-analysis has indicated that Tai Chi is a worthy complementary non-pharmacological resource which works by ameliorating depression and anxiety and may have great implications in public health (Zhang et al., 2019). However, the effects of Tai Chi on the anxiety and depression of patients with CHD after PCI and its related molecular mechanisms remain unclear.

miRNAs play important roles in the symptoms of depression (Ma et al., 2019) and anxiety (Du et al., 2019). miRNAs are non-coding RNA molecules with 18- to 28-bp nucleotides. They play critical roles in the posttranscriptional regulation of protein expression, which is involved with the normal and pathological cellular processes, including cell differentiation, cell cycle progression, and apoptosis (Tanase et al., 2012). miRNAs can affect the expression of various genes and may be the candidates associated with depression symptoms (Yuan et al., 2018). Tai Chi has been found to exert a protective function by affecting the serum levels of miRNA (Li et al., 2019) and may also improve depression and anxiety symptoms by affecting the miRNA.

The miR-17-92 cluster host gene spans 7 kb and contains the 800-nucleotide cluster transcript that encodes miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1. The miR-17-92 cluster is an important indicator in the development of the immune system, the heart, and the lung and in oncogenic patients. It is involved with cellular innate and adaptive immunity, such as B cells, T-lymphocyte subsets, T follicular helper cells, regulatory T cells, monocytes, macrophages, and so on (Kuo et al., 2019). The miR-17-92 cluster plays a critical role in cardiomyocyte proliferation (Gao et al., 2019) and affects the development of various diseases by regulating many related cellular processes and multiple target genes, including neurological diseases and cardiac diseases (Bai et al., 2019). The members of the miR-17-92 cluster and the expression of their passenger miRNAs expressions promote exercise-induced cardiac growth and reduce adverse ventricular remodeling (Shi et al., 2017). Cardiac rehabilitation will contribute in protecting against the development of depression and anxiety (Zheng et al., 2019). A recent study showed that miR-17-92 may regulate neurogenesis and anxiety- and depression-like behaviors (Jin et al., 2016). Tai Chi can affect the level of miRNA, and it possibly also affects the level of miR-17-92 because it can improve the symptoms of anxiety and depression (Rawtaer et al., 2015).

There are four major styles of Tai Chi, including Chen style (Zou et al., 2019), Yang style (also 24-form style) (Zou et al., 2017), Sun style (Liu and Cao, 2018), and Wu style (Taylor-Piliae et al., 2012). The former styles are effective in improving global cognitive function, balance, and fitness (Zou et al., 2019). However, Chen style is more complex than Yang style; thus, the latter style of Tai Chi (24-form) was tried in the present work. The present paper aims to investigate the effects of the 24-form Tai Chi on the anxiety and the depression of CHD patients after PCI.

MATERIALS AND METHODS

Calculation of Sample Size

The sample size was calculated according to the following equation: $n = (\mu_\alpha + \mu_\beta)^2 (1 + 1/k) \sigma^2/\delta^2$ according to a previous report (Nyklicek et al., 2014), where $\alpha = 0.05$, $\beta = 0.2$, $\sigma = 0.45$, and $\delta = 0.38$. δ stands for the smallest difference and $\delta = 0.38$ will be detected when power is 0.8. The calculated sample size was 32. Considering 10% loss of follow-up, the sample size per group was 35.

Inclusion Criteria

Before the present experiment, all processes were approved by the Human Research Ethics Committee of The First Hospital of Jilin University (Approval No. JLUFH-2468HD). The patients were included if they had one of the following criteria: (1) anxiety, depression, angina and dizziness, asthma, chills, sweating, nausea, and even syncope and other clinical symptoms; (2) coronary angiography showing that one of the three main coronary or left main porridge sclerosing lesions and luminal stenosis was more than 50%; (3) history of acute myocardial infarction and ECG showing old infarct Q waves; and (4) ST segment of ECG at rest or following exercise was horizontal

or down-tilted lower than 1 mm, and continuous time was more than 2 min. PCI stent was implanted 1–4 days ago, and the patients agreed to sign an informed consent stating their willingness to participate in the study.

Exclusion Criteria

The patients were excluded if they met one of following items: age over 70 years old and the treatment for mental illness and severe physical and psychological complications such as cancer, mental illness, and brain damage.

General Questionnaire

The general questionnaire was created and modified according to a previous report (Akbaraly et al., 2015) and our demographic and clinical data. The general survey questionnaire included demographic data such as age, gender, height, weight, disease diagnosis, and PCI indication and clinical data such as the number of diseased blood vessels, stents, comorbidities, family history of cardiovascular disease, myocardial infarction, and medication being used. The anxiety scores were measured by using the General Anxiety Disorder 7-Item (GAD-7) (Johnson et al., 2019). The GAD-7 scores were classified as 0–4 (no anxiety symptoms), 5–9 (mild), 10–14 (moderate), and 15–21 (severe). Depression scores were measured by using the Patient Health Questionnaire-9 (PHQ-9) (Levis et al., 2019). The PHQ-9 scores were classified as 0–4 (no depressive symptoms), 5–9 (mild), 10–14 (moderate), 15–19 (moderately severe), and 20–27 (severe). All anxiety and depression questionnaires were asked on the Monday, Thursday, and Sunday within the week before intervention or prior to the end of intervention, and the mean scores were calculated.

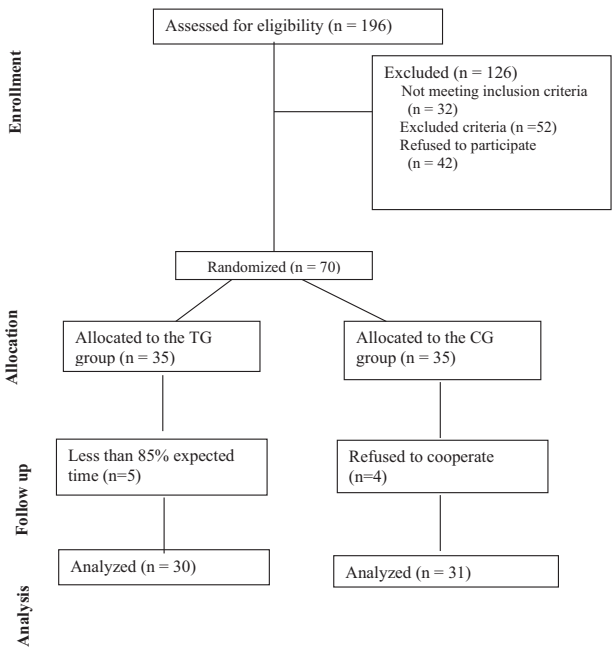

FIGURE 1 | Consort diagram of the present study. The enrolled patients were numbered according to the patients' admission order and randomly divided into the Tai Chi group (TG) and the control group (CG) by using a random number table. The duration of follow-up was 10 months.

Hospital Anxiety and Depression Scale (Zigmond and Snaith, 1983)

The scale has 14 items, including sub-scales of anxiety and depression (seven items each), respectively. Points for each item are 0–3, and the highest scores for anxiety and depression are no

TABLE 1 | Baseline characteristics between the two groups.

Characteristics	CG (*n* = 31)	TG (*n* = 30)	*P*-values
Age, years	56.97 ± 10.98	60.40 ± 10.86	0.225
Sex, male (%)	25 (83.33)	24 (77.42)	0.561
BMI, kg/m^2	20.52 ± 2.46	25.19 ± 3.90	0.418
Anxiety, cases (%)	17 (54.80)	15 (50.00)	0.684
Anxiety scores	5.98 ± 3.87	6.04 ± 4.21	0.653
Depression, cases (%)	17 (54.80)	18 (60.00)	0.705
Depression scores	7.23 ± 5.46	6.91 ± 5.24	0.418
HADS	15.67 ± 5.42	16.94 ± 6.66	0.815
A	7.80 ± 3.00	8.45 ± 3.20	0.820
D	8.00 ± 3.59	8.77 ± 4.29	0.763
Stress scores	47.15 ± 6.08	47.76 ± 5.83	0.512
Diagnosis			
STEMI	11 (35.48)	10 (33.33)	0.860
Non-STEMI	2 (6.45)	3 (10.00)	0.969
Unstable angina	10 (32.26)	10 (33.33)	0.929
Stable angina	8 (25.81)	7 (23.33)	0.823
PCI indication			
Emergency PCI	10 (32.26)	8 (26.67)	0.632
Elective PCI	21 (67.74)	22 (73.33)	
Number of diseased vessels	2.00 ± 0.73	1.80 ± 0.96	0.365
Number of stents	1.42 ± 0.62	1.51 ± 0.82	0.430
Type of medicine	5.58 ± 1.57	5.47 ± 1.20	0.751
Hypertension	13 (41.94)	18 (60.00)	0.158
Diabetes	8 (25.81)	7 (23.33)	0.823
Hyperlipidemia	1 (3.23)	3 (10.00)	0.354
Family history of early-onset coronary heart disease	0 (0.00)	1 (3.33)	0.492
Heart attack	4 (12.90)	3 (10.00)	1.000
Seeking psychological counseling	0 (0.00)	0 (0.00)	NS

Note: All data are expressed as n (%) or mean ± standard deviation. NS, no statistical data; BMI, body mass index; SETM, ST-segment elevation myocardial infarction; non-STEMI, non-ST-segment elevation myocardial infarction. The statistical difference was significant if P < 0.05.

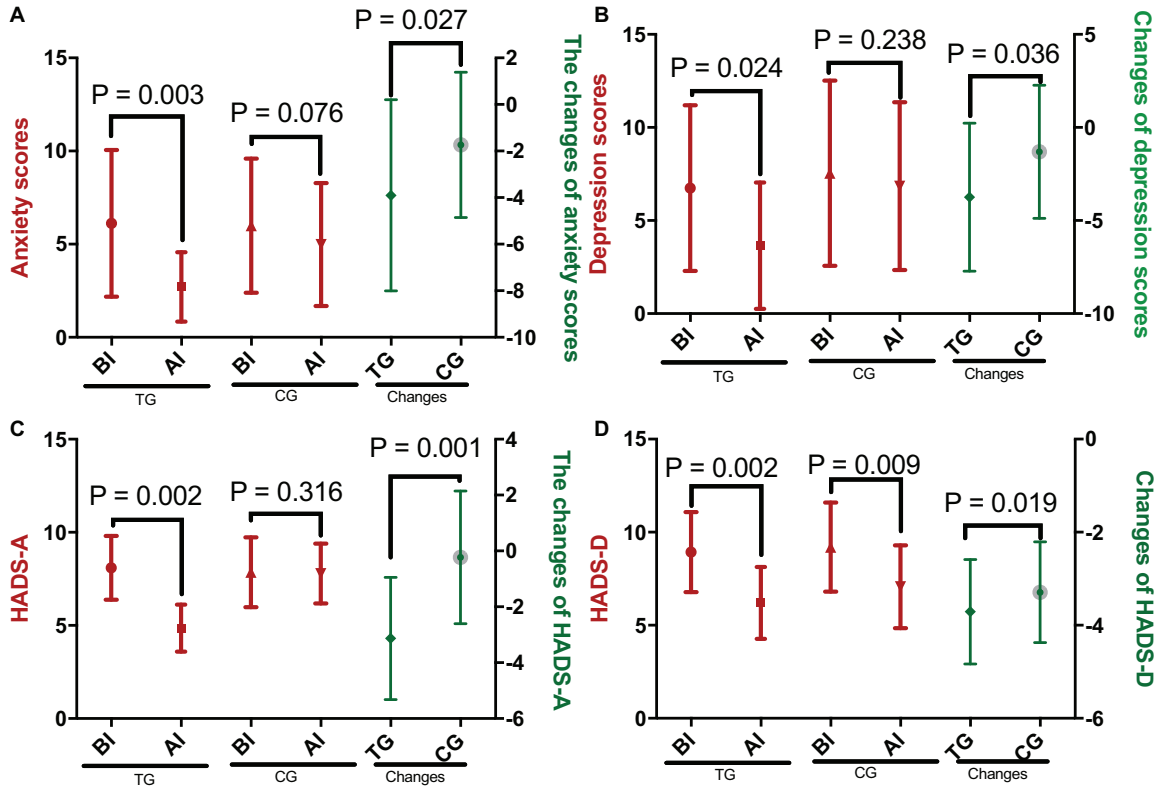

FIGURE 2 | The effects of Tai Chi on anxiety and depression scores. **(A)** Anxiety scores, **(B)** depression scores, **(C)** HADS-A, and **(D)** HADS-D. The enrolled patients were numbered according to the patients' admission order and randomly divided into the Tai Chi group (TG, $n = 30$) and the control group (CG, $n = 31$). The changes stand for the difference between the scores obtained before and after intervention for both the Tai Chi and the control groups. The duration of follow-up was 10 months. AI, after intervention; BI, before intervention. The statistical difference was significant if $P < 0.05$.

more than 21 points, whereas the lowest score is 0. The anxiety subscales are 1, 3, 5, 7, 9, 11, and 13; the depression subscales are 2, 4, 6, 8, 10, 12, and 14. Among the anxiety subscales, the seventh item is scored in reverse, and the 2nd, 4th, 6th, 12th, and 14th items in the depression subscale are reversely scored. According to the original author's criteria, the total score of the two subscales is 0–7, which means no anxiety or depression; a total score of 8–10 represents possible or critical anxiety or depression, and a total score of 11–21 points may indicate serious anxiety or depression.

Perceived Stress Scale (Cohen et al., 1983)

The scale consists of 14 items, and each item has five points and reflects stress and sense of control. Seven out of the 14 items of PSS-14 are considered as negative (1, 2, 3, 8, 11, 12, and 14) and the remaining seven as positive (4, 5, 6, 7, 9, 10, and 13), representing perceived helplessness and self-efficacy, respectively. Each item was rated on a five-point Likert-type scale (0 = never to 4 = very often). The total scores are calculated after reversing the positive items' scores and then summing up all scores. The total scores for PSS-14 range from 0 to 56. A higher score indicates greater stress. The scale was used to evaluate three stress scenarios: (1) daily chores, (2) major events, and (3) changes in stressors. The respondent answers the stress situation in the past month. The data showed good reliability and validity with the Cronbach's α coefficient of 0.78. The correlation coefficient between the items was 0.28 on average, and the correlation coefficient between the total scores was 0.37–0.53. According to a previous report, Cronbach's α coefficient of 0.78–0.88 indicates an adequate internal consistency (Jauregui-Lobera et al., 2011) and the inter-item correlation for the optimal level of homogeneity ranges from 0.2 to 0.4 (Briggs and Cheek, 1986), and so the present values showed high internal consistency and homogeneity.

Measurement of Life Quality

The life quality of the CHD patients after PCI was measured by using the Medical Outcomes Study 36-item Short-Form Health Survey (SF-36) version 2, which consists of assessing PF (10 items), SF (two items), RP (four items), BP (two items), MH (five items), RE (three items), VT (four items), and GH (five items) (Ware, 2000).

Patient Grouping

This is a single-blinded study, and informed consent was obtained from the patients who were eligible for the study within 1–4 days after PCI and who met the inclusion and exclusion criteria. Allocation concealment was performed to

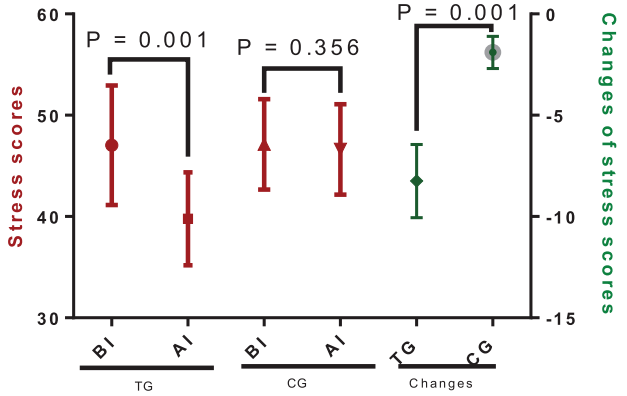

FIGURE 3 | The effects of Tai Chi on stress scores. The enrolled patients were numbered according to the patients' admission order and randomly divided into the Tai Chi group (TG, $n = 30$) and the control group (CG, $n = 31$). The changes stand for the difference between the scores obtained before and after intervention for both the Tai Chi and the control groups. The duration of follow-up was 10 months. AI, after intervention; BI, before intervention. The statistical difference was significant if $P < 0.05$.

avoid subsequent interference. The enrolled patients were numbered according to the patient's admission order and randomly divided into the 24-form Tai Chi group (TG) and the control group (CG) with equal allocation ratio by using a random number table generated by a computer. All CHD patients in the CG group received routine treatment, examination, nursing, and health education. The antidepressant amitriptyline was administered at a dose of 50–200 mg/day according to the different severity degrees of depression. The TG group received the same treatment as the CG group. Meanwhile, the 24-form Tai Chi exercise was provided with reference to the Health Qigong 24-form Taijiquan issued by the State Sports General Administration in 2003 and developed by recent work (Lee, 2017; Zou et al., 2017; Deng and Xia, 2018), performed two times per day starting from 6:00 to 8:00 am and from 16:00 to 17:00 pm at 50–60 min per session. According to the individual condition of the patient, the exercise intensity and the exercise volume should be adjusted according to the patient's individual condition, and patient tolerance should be appropriate.

Real-Time qPCR Analysis of miR-17-92

A sample of 5 ml of venous blood was taken from each subject under aseptic precautions, and the serum was prepared through centrifugation at 1,000 (×g for 10 min. All of the sera were collected at the time of diagnosis and the total RNA was extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen Inc., CA, United States). Reverse transcription-related operation was performed by using Qiagen's QuantiTect Reverse Transcription Kit. The relative level of miR-17-92 was measured by using the SYBR Premix Ex Taq TM (TaKaRa, Dalian, China). The primers for miR-17-92 (forward primer 5′-TCATACACGTGGACCTAAC-3′ and reverse primer 5′-CTCTCTAAGAAACCAATCC-3′) and U6 (forward primer 5′-GCTTCGGCAGCACATATACTAAAAT-3′ and reverse primer 5′-CGCTTCACGAATTTGCGTGTCAT-3′) were synthesized by TaKaRa. The PCR was performed as follows: 95°C for 3 min, one cycle and 45 cycles of 20 s at 94°C and 40 s at 60°C, respectively. Default threshold settings were used as threshold cycle (Ct). After calculating the Ct value, the level of miR-17-92 was normalized to U6, and the relative quantification of miRNA expression was calculated with $2\text{-}\Delta\Delta Ct$. The miRNA stability was measured by using the total RNA harvested at 4, 8, and 12 h (Mall et al., 2013), and the miRNA expression was confirmed using the abovementioned real-time qPCR.

Statistical Analysis

Data analysis was performed using the SPSS 20.0 statistical software. The data were expressed as mean value ± SD (standard deviation). The count data were analyzed by chi-square test. The Kolmogorov–Smirnov (KS) and the Shapiro–Wilk tests were used to test the normal distribution of all variables (Nguyen et al.,

TABLE 2 | The effects of Tai Chi on the life quality of CHD patients after PCI.

Variables	TG, $n = 30$		*P*-values	CG, $n = 31$		*P*-values	Change over study period		
	Before intervention	After intervention		Before intervention	After intervention		TG	CG	*P*-values
PF	56.8 ± 9.6	86.6 ± 8.5	0.001	56.1 ± 9.2	72.3 ± 7.2	0.001	35.12 ± 5.58	17.46 ± 3.69	0.011
RP	64.1 ± 8.0	89.9 ± 7.4	0.001	63.2 ± 8.1	74.2 ± 6.9	0.001	33.72 ± 4.50	13.18 ± 4.90	0.002
BP	72.6 ± 8.2	86.8 ± 8.2	0.018	72.0 ± 8.5	72.5 ± 7.9	0.892	16.31 ± 1.75	0.98 ± 0.23	0.023
SF	75.1 ± 8.2	79.5 ± 8.2	0.078	74.2 ± 8.3	68.1 ± 8.0	0.012	6.24 ± 0.79	−6.15 ± 1.16	0.003
VT	58.0 ± 9.1	88.7 ± 8.5	0.001	57.3 ± 9.4	70.6 ± 7.6	0.010	31.24 ± 1.45	13.18 ± 3.15	0.001
RE	71.3 ± 9.6	79.6 ± 8.1	0.039	72.4 ± 8.1	64.9 ± 7.2	0.005	8.47 ± 1.93	− 8.12 ± 1.44	0.002
MH	57.3 ± 8.2	85.2 ± 7.9	0.001	56.4 ± 8.3	70.3 ± 8.3	0.001	29.34 ± 1.68	14.52 ± 1.19	0.001
GH	63.1 ± 8.2	87.8 ± 7.1	0.001	62.5 ± 8.0	72.4 ± 7.0	0.003	24.77 ± 3.63	11.64 ± 1.65	0.003

Note: The SF-36 version 2 consists of assessing physical functioning (PF; 10 items), social functioning (SF; two items), role limitation due to physical health (RP; four items), bodily pain (BP; two items), mental health (MH; five items), role limitations due to emotional health (RE; three items), vitality (VT; four items), and general health perceptions (GH; five items) (Ware, 2000). Adjusted Cox proportional hazards models were used to assess the association between the 24-form Tai Chi and the clinical outcome stratified by depression, anxiety, and SF-36 including age, gender, BMI, angina, medicine, PCI, heart attack, and family history of CHD. The statistical difference was significant if $P < 0.05$.

2013). The two-sided *t*-test was used to compare the anxiety, depression, stress, and SF-36 scores before and after the test. The Mann–Whitney *U*-test was conducted instead when the condition of normality was not met (Perme and Manevski, 2019). The correlation between anxiety or depression or stress and Tai Chi was analyzed using the Pearson correlation coefficient test (Liu et al., 2017). Anxiety and depression were used as dependent variables, and the Tai Chi exercise was used as the independent variable. Adjusted Cox proportional hazard models were used to assess the association between the 24-form Tai Chi and clinical outcome stratified by stress, anxiety, and SF-36 including age, gender, body mass index (BMI), angina, medicine, PCI, heart attack, and family history of CHD. The test level was bilaterally $P < 0.05$ considered as statistically significant.

RESULTS

Study Participant Demographics and Baseline Characteristics

From 1 March 2016 to 1 June 2017, 196 patients with CHD stent implantation visited the First Affiliated Hospital of Jinlin University; 112 of the patients met the inclusion criteria, 42 patients refused to participate, and eventually 70 patients were included. The patients were randomly divided into the TG and the CG groups ($n = 35$ for each group). During the trial, there were five patients who joined in the Tai Chi practice less than 80% of the expected time and recorded as "exit" in the TG group, and four patients in the CG group refused to cooperate with the unfinished indicator collection. Finally, 61 patients completed all interventions and data collection, 30 patients from the TG group and 31 patients from the CG group (**Figure 1**). The baseline parameters were adjusted for differences by using propensity score matching to reduce the influences of possible confounders and of collection bias. The statistical differences for the number of anxiety and depression and the stress scores were not significantly different between the two groups (**Table 1**, $P > 0.05$). The statistical difference was not significantly different for the baseline levels of anxiety scores, depression scores, and HADS levels, both subscale HADS-A and subscale HADS-D, between the TG and the CG groups (**Table 1**, $P > 0.05$). No statistical difference was observed for the other parameters between the two groups ($P > 0.05$, **Table 1**) after an independent sample *t*-test or χ^2 test.

Intervention Effects

Tai Chi Intervention Reduced the Severity of Depression and Anxiety

Tai Chi intervention time was 290–360 days, with an average time of 300.9 days. The completion rate of the intervention program was 80.56–100%, with an average of 85.93%.

The anxiety (**Figure 2A**) and the depression (**Figure 2B**) scores and the HADS-A (**Figure 2C**) and the HADS-D (**Figure 2D**) values were significantly decreased over the study period in the TG. On the other hand, only the HADS-D values were significantly decreased in the control group (CG; **Figure 2D**). Changes in the anxiety and the depression scores and in the HADS-A and the HADS-D values were significantly higher in the TG when compared with those of the CG (**Figure 2**, $P < 0.05$). The results suggested that Tai Chi intervention improved the symptoms of anxiety and depression in the CHD patients.

Tai Chi Intervention Reduced the Stress of CHD Patients After PCI

The stress scores were significantly decreased over the study period in the TG (**Figure 3**, $P = 0.001$) but not in the CG (**Figure 3**, $P = 0.351$). Additionally, changes in the stress scores were significantly higher in the TG when compared with those of the CG (**Figure 3**, $P = 0.001$). The results suggested that Tai Chi intervention improved the symptoms of anxiety and depression in the CHD patients.

Tai Chi Intervention Improved the Life Quality of CHD Patients After PCI

The statistical difference for SF-36 scores was not significantly different between the two groups before Tai Chi intervention ($P > 0.05$, **Table 2**). The SF-36 scores in the TG group were higher than in the CG group after Tai Chi intervention ($P < 0.05$, **Table 2**). These results suggested that Tai Chi improved the life quality of CHD patients after PCI.

Tai Chi Intervention Increased the Serum Level of miR-17-92

The relative levels of miR-17-92 were significantly increased over the study period in the TG (**Figure 4**, $P = 0.001$) but were not statistically different in the CG (**Figure 3**, $P = 0.305$). Moreover, changes in the relative levels of miR-17-92 were significantly higher in the TG when compared with those of the CG (**Figure 4**, $P = 0.001$). The results suggested that Tai Chi exercise increased the serum level of miR-17-92 as revealed by the change.

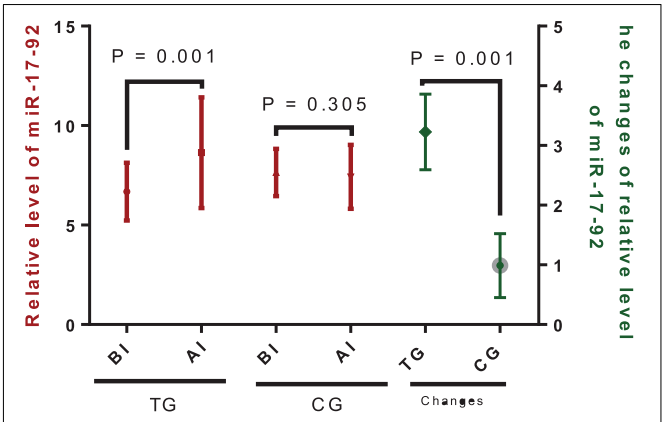

FIGURE 4 | Relative levels of miR-17-92 between the Tai Chi (TG) and the control (CG) groups. The enrolled patients were numbered according to the patients' admission order and randomly divided into the Tai Chi group ($n = 30$) and the control group ($n = 31$). The changes stand for the difference between the scores obtained before and after intervention for both the Tai Chi and the control groups. The duration of follow-up was 10 months. AI, after intervention; BI, before intervention. The statistical difference was significant if $P < 0.05$.

Correlation Between miR-17-92 and Emotional Disorders

Pearson correlation coefficient test showed that, with the increase in the serum levels of miR-17-92, the levels of anxiety (**Figure 5A**), the scores for depression (**Figure 5B**), and the stress scores (**Figure 5C**) were reduced significantly ($P < 0.001$). The serum level of miR-17-92 had negative correlation with anxiety, depression, and stress scores since rho < -0.5 and $P < 0.001$.

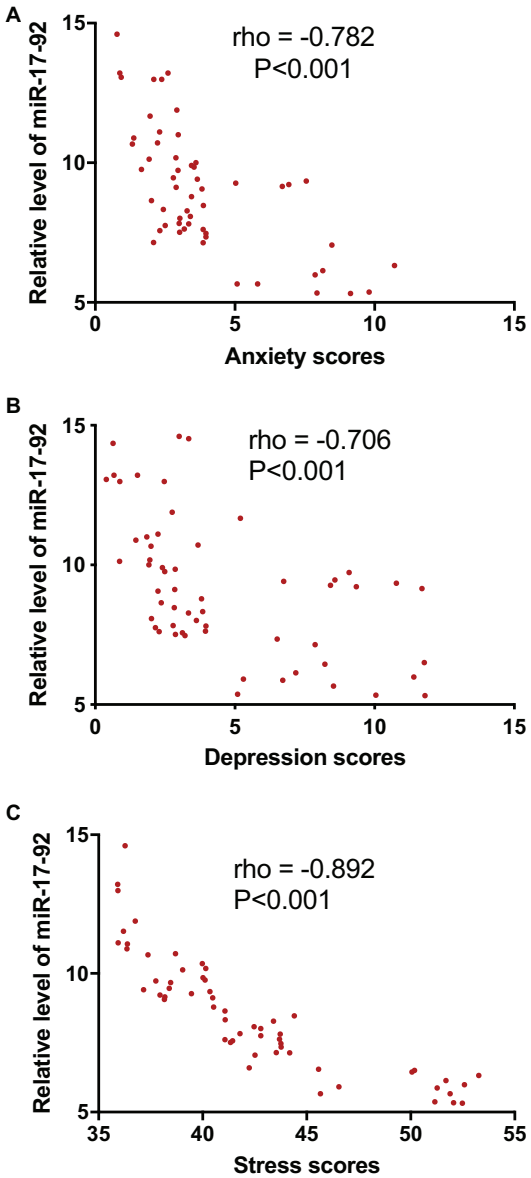

FIGURE 5 | The correlation between the relative levels of miR-17-92 and anxiety, depression, and stress scores. **(A)** The correlation between the relative levels of miR-17-92 and the anxiety scores. **(B)** The correlation between the relative levels of miR-17-92 and the depression scores. **(C)** The correlation between the relative levels of miR-17-92 and the stress scores. There was a strong negative correlation between the two variables if rho values $< --0.5$.

DISCUSSION

According to the GAD-7 and the PHQ-9 score assessments, there were 17 patients with anxiety and 17 patients with depression in the CG group and 15 patients with anxiety and 18 patients with depression in the TG group before intervention (**Table 1**, $P = 0.684$ and 0.705, respectively). Comparatively, there were 11 patients with anxiety and 14 patients with depression in the CG group and four patients with anxiety and seven patients with depression in the TG group after intervention ($P = 0.045$ and 0.073, respectively). In a similar case, the statistical difference was not significantly different for the stress score before intervention (**Table 1**, $P = 0.512$), and the statistical difference was significant after intervention ($P = 0.001$). Most patients in both groups had mild anxiety and depression (according to the GAD-7 and the PHQ-9 score assessments), in which the patients experienced instability in emotionality, relationships, and behavior. The anxiety and depression symptoms were further measured by the HADS. Both evaluations showed consistency. The perceived stress was associated with a CHD incident and typical symptoms included worry and rumination. The patients further exhibited sleep problems.

This study also showed that there was no significant difference in HADS score, anxiety subscale score, depression subscale score, and the proportion of the patients with anxiety or depression and stress between the two groups before intervention. The findings demonstrated that Tai Chi improved the depression and the anxiety symptoms and reduced the pressure of self-perception in the CHD patients after PCD and was not affected by baseline characteristics.

Tai Chi exercise can significantly improve the quality of life of patients. Other studies have also confirmed that Tai Chi can effectively improve the patient's circulation and balance, can help relax and strengthen the nervous system (Zhang, 2015a), and can lead to good regulation of the cardiovascular system (Liu et al., 2018) and the respiratory system (Song et al., 2014; Siu, 2016) to effectively improve the quality of life of the patients.

Tai Chi improved the symptoms of emotional disorders in patients with CHD after PCI. Tai Chi is combined mind and physical exercise and may be more effective than aerobic exercise in preventing some diseases (Ostrovsky, 2018).

The critical role of miRNA dysregulation in psychiatric disorders has been widely reported. For instance, preclinical evidence supports that the microRNA-34 family is involved in stress-related psychiatric conditions and in the modulation of depression (Lo Iacono et al., 2019). miR-140-5p has been found to be associated with the pathogenesis of late-onset post-stroke depression (Liang et al., 2019). The miR-101a-3p and its target, enhancer of zeste homolog 2 (Ezh2) in the amygdala, contribute to anxiety-like behavior (Cohen et al., 2017). The microRNA levels may change based on the training type or exercise (Domanska-Senderowska et al., 2019). Aerobic exercise delays neurodegenerative diseases and lesions by regulating miR-3557/324 (Liu et al., 2019). It is quite possible that Tai Chi also induces the changes in miRNA. The present work also showed

that the serum level of miR-17-92 was increased after Tai Chi intervention. Furthermore, a negative correlation between the serum level of miR-17-92 and the scores of anxiety, depression, and stress was observed (**Figure 5**). The results suggested that the miR-17-92 changes may contribute to the emotional changes.

There were some limitations in the present study: only the levels of anxiety, depression, stress, and SF-36 were measured in the CHD patients after PCI within 1 year and repeated evaluations were not performed until 1 year later, so the persistent effects of Tai Chi on adverse cardiovascular events were not determined. "HADS" was used to assess the patients' anxiety and depression, and psychological interviews were not conducted. The exact molecular mechanism for the functional role of Tai Chi was not explored in the CHD patients after PCI. The possible side effects of Tai Chi were not investigated, although most reports showed that Tai Chi could reduce most side effects of various diseases (Murley et al., 2019; So et al., 2019). According to our experiences, there is a difference in learning Tai Chi between males and females, but the issue was not explored in the present study. Further work is highly needed in the future.

CONCLUSION

Tai Chi improved the symptoms of anxiety, depression, and stress and upregulated the miR-17-92 in CHD patients after PCI. Tai Chi also improved the quality of life of the CHD patients. This is suggestive that Tai Chi should be used as a potential way to improve the emotional parameters of the CHD patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by this Ethics Committee of The First Hospital of Jilin University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WL and XW designed the study, performed the experiment, and revised the manuscript. JL and PY performed the experiment and wrote the manuscript. All authors read and approved the final manuscript.

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