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Basic and translation research in learning and memory

Edited by
Adebobola Imeh-Nathaniel, Lauren A. Fowler and Sylvester Olubolu Orimaye

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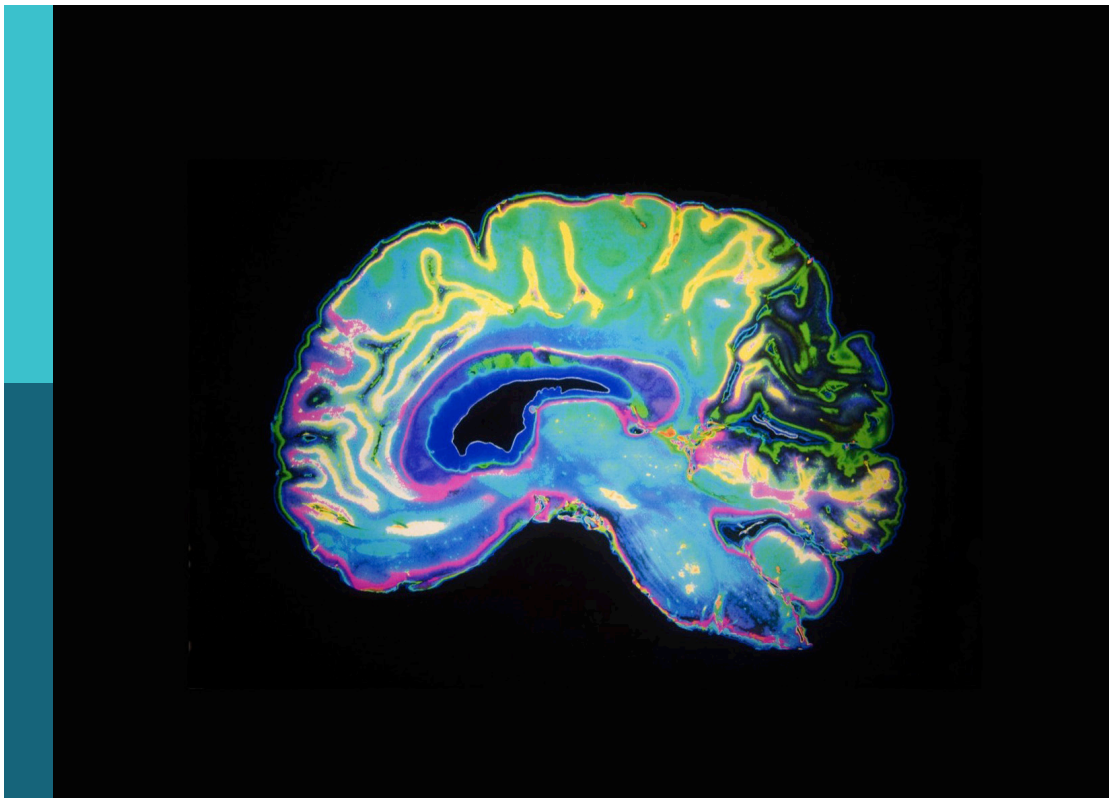

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Basic and translation research in learning and memory

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Early Event-Related Potential During Figure and Object Perception of Abacus Mental Calculation Training Children: A Randomized Controlled Trial

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The aim of this study was to discuss the effect of abacus mental calculation (AMC) on the early processing of children's perception on numbers and objects. We designed a randomized controlled trial, and a total of 28 subjects were randomly distributed into two groups of equal numbers, namely, one group that received AMC training (training group) and the other group that did not receive training (non-training group). The subjects were asked to determine the figures and objects shown on the computer screen and were recorded on the computer. The event-related potential (ERP) component (N1, N170, P1, and P2) of different brain areas between the two subject groups was compared. Compared with the non-training group, the training group's P1 in the occipital region showed a larger amplitude and a longer potential period. For N1, the training group showed a longer potential period. Additionally, for N170, the training group showed a smaller amplitude. Finally, the observation of P2 showed a smaller amplitude in the training group and a longer potential period in the condition of object stimulus. Overall, the activated degree of the occipital region of children who received AMC training was enhanced, while the activated degree of the central region of the forehead and temporal occipital region was slightly down. Meanwhile, the potential periods of all components were extended. Therefore, long-term AMC training can change children's cortical function activities.

Keywords: event-related potential, abacus mental calculation, figure perception, object perception, children, cognitive capacity

INTRODUCTION

Abacus mental calculation (AMC) is a cognitive skill based on abacus' use to form bead-image movement in the brain through actual bead-driven training, simulated bead-driven training, and image bead-driven training. It is an advanced calculation function of the brain. AMC training can make a person solve mathematical problems more accurately and quickly (Hu et al., 2011). There are three stages to acquire this capability: first, one should learn to calculate using a real abacus (a simple device consisting of beads and rods); second, after becoming familiar with the operation

of the abacus, he/she will be instructed to imagine moving the beads in his/her mind with actual finger movements to finish the calculation; and third, he/she can try to calculate *via* the imaginary abacus completely. AMC can improve many aspects of cognition (Hanakawa et al., 2003). Previous studies have found that AMC can not only improve the efficiency of mental calculation of children but also enhance children's attention, memory, thinking, and various basic cognitive abilities (Hatta and Miyazaki, 1989; Liu and Sun, 2017). In addition to cognitive improvements, AMC training was also found to improve activation levels and neuroplasticity in some brain regions (Czigler and Csibra, 1990; Stiles, 2000; Tanaka et al., 2012; Weng et al., 2017) can also be found. Conversely, some studies have found that AMC training cannot improve cognitive abilities. A study by Barner et al. (2016) involving 183 children over 3 years reported that AMC training provided no benefit for basic cognitive abilities. A study by Xie et al. (2018) assessing 162 children for 1 year supported the finding that abacus arithmetic had a slight effect on children's memory only in the early stages of training, while no significant difference was observed in the memory performance between the training group and the non-training group after training. So, the influence of AMC on cognition should be further explored. Perception is considered as a basis for the superior cognitive stage and regulates the relationship between the body, the environment, and related behaviors (Dong and Bao, 2021). This study aims to adopt event-related potential (ERP) technology to investigate the impact of AMC on the early processing of children's perception on numbers and objects from the angle of sensory perception.

We hypothesized that the training group was able to process digital stimuli with less brain activation, which was represented by a reduction in the amplitude of the ERP component. In addition, AMC training can improve children's early attention to visual stimulus, which is manifested by a longer potential period of ERP components.

MATERIALS AND METHODS

The Objects of Study

The experiment was designed with a randomized control trial. The samples were randomly selected from students of grades 3 and 4 at Bei Guan Central Primary School in Weifang from March to April 2020. A total of 28 students (14 boys and 14 girls) participated in the study. They were randomly divided into two groups, namely, 14 children who received AMC training (training group) (starting from the first grade of primary school, two 50-min sessions a week for 20 weeks) and other 14 children who did not receive training (non-training group); there were 7 boys and 7 girls in both groups. The participants aged 10–11 years, with an average age of 10.5 years, and the average age was 10.4 years in the training group, whereas it was 10.6 years in the non-training group. There were no significant differences in age, family background, educational background, and score between the two groups. The students participated in this ERP experiment for the first time. Since the difference in handedness might lead to different active patterns of the brain, we selected only right-handed participants to make the ERP's result comparable. In addition, all participants had visual acuity or corrected visual acuity of 1.0 or higher. This study was approved by the Ethics Committee of Beijing Chaoyang Hospital, and all subjects have signed informed consent.

Experimental Instrument

A 64-channel NeuroScan ERP workstation made in the United States was used. The electrodes were set according to the international 10/20 system standard (Xu et al., 2012). The reference electrodes were placed at the right and left mastoids, and the ground electrode was placed 1 cm under the forehead hairline. At the same time, both horizontal and vertical electrooculography (EOGs) were recorded. Filtering bandpass was 0.05–40 Hz. The sampling rate was 1,000 HZ/channel, and the scalp resistance was less than 5 kΩ. A standard 32-channel electrode cap for electroencephalogram (EEG) was used to record the electrical activity of the brain.

Materials for the Simulated and Experimental Procedure

The stimulation consisted of two types. The first type of stimulation was a number; 3–8 numbers were randomly arranged and individually shown on the computer screen. The second type of stimulation was composed of circle-representative objects, numbering 3–8 (**Figure 1**). Stim2 was used to write a stimulus program. The stimulation was randomly arranged. Each stimulus was shown for 500 ms with a stimulus interval (ISI) of 500 ms. The stimulus was repeated 10 times for a total of 120 times. The stimulation was shown on a 15-foot computer screen. The background was black, and the numbers or circles were white. The subjects of the experiment sat on chairs, facing the display screen. The sight distance was 60 cm. At the start of the experiment, the subjects were required to look at the point of fixation (a cross in white). When the stimulus appeared, the students were told to pay more attention to observing the shown numbers and pictures. When the numbers appeared, the students were asked to press the digit 1 button on the key box, while the object appeared, they were asked to press the digit 2 button. To eliminate the influence of left- and right-hand buttons on the computer, the selection of buttons was cross-balanced among the students.

During the experiment, the focus was on the changes in EEG. The experiment results were saved once the experiment was completed. EEG records were then added to calculate the average. The reference values of right and left mastoids were converted to the average reference voltage to make corrections, excluding wink, eye movement, EMG, and other artifacts. The analytic window of EEG was −100–500 ms, and −100–0 ms was used as the baseline to make corrections. Through classification and addition, two ERPs were caused by different stimuli, and two types of ERPs were caused by different subjects. Given the purpose of this experiment, several electrode points in the temporal occipital region (P7/P8), the occipital region (O1, OZ, and O2), and the central region of the forehead (FZ, FCZ,

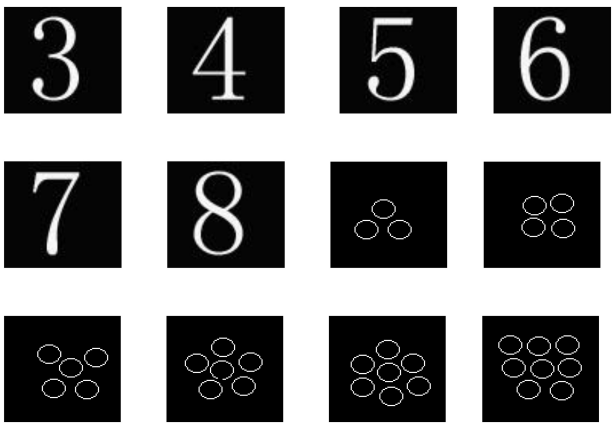

FIGURE 1 | Pictures of number and object-controlled experiments.

and CZ) were used as the representative points in the analytic position. The analysis time window of P100 at the real scalp was 50–150 ms; the analysis time window of N170 was 140–220 ms; the analysis time window of P2 at the fore scale was 180–270 ms; and the analysis time window of N1 was 75–150 ms. The measurement methods in peak amplitude and peak potential periods were used. The results were filtered using the zero phase with a bandwidth of 0.8–30 Hz.

STATISTICAL ANALYSES

The SPSS statistical software was used in the 3-factor analysis of variance on the aforementioned amplitudes and potential periods. The between-subject factor refers to the type of subject (two levels, i.e., the training group and the non-training group). The within-subject factor refers to the type of stimulus (two levels: figure and circle) and the positions of electrodes (rear P1: three levels; N170: two levels; and front N1, P2: three levels). We used the SPSS17.0 to analyze and process the data. The Greenhouse-Geisser method was used to correct the *P*-value.

RESULTS

Features of Event-Related Potentials' Early Component

As shown in **Figures 2–4** (ms refers to millisecond and μV refers to microvolt), the ERPs of the two groups caused by different stimuli evoke consistency on the basic features. A general visual-evoked response can be observed, i.e., P1 mainly composed of the occipital region (O1, OZ, and O2); N1 and P2 were in the forehead central area (FZ, FCZ, and CZ); N170 was in the temporal occipital region (P7 and P8). However, when the two groups were compared, significant differences in the amplitudes were observed. The P1 amplitude of the training group was larger than the non-training group, especially the P1 amplitudes of the training group caused by the figure was larger than that of the non-training group, while in each group, the difference of P1 amplitudes caused by object stimulus was not significant. The P1 potential period of the training group was longer than that of the non-training group. No significant difference existed between the N1 amplitude of the training group and that of the non-training group; the N1 potential period of the training group was longer than that of the non-training group. The N170 amplitudes of the training group were smaller than those of the non-training group, especially in the P7 electrode position; the N170 potential period of the training group was longer than that of the non-training group. The P2 amplitude of the training group was smaller than that of the non-training group; the P2 potential period of the training group was longer than that of the non-training group, especially under the condition of object stimulus.

Comparison of the Occipital Region P1 Amplitudes and the Potential Periods Between the Two Groups Under Different Stimulation Conditions

The interaction between the positions of the electrodes of occipital region P1 amplitudes and the types of subjects was significant ($F = 12.69$, $P < 0.01$). After the interaction of the two factors was found, we subsequently conducted a simple effect analysis. We hoped to further analyze the different levels of factors that have a significant effect at a certain level of another factor. Further simple effect analysis showed that the main effect in the type of subject was significant in the left-brain area (O1) ($F = 5.36$, $P < 0.05$). This indicates that the training group has an impact on the amplitude of the P1 component, and this effect is significant at the O1 electrode position. The P1 amplitudes of the training group were larger than those of the non-training group. The main effect in the type of stimulus was significant ($F = 49.85$, $P < 0.01$). The interaction between the type of stimulus and the type of subject was significant ($F = 30.35$, $P < 0.01$). Further simple effect analysis showed that, under the condition of numerical stimulus, the main effect of the type of subject was significant ($F = 4.85$, $P < 0.05$). The occipital region P1 amplitudes of the training group were larger than those of the non-training group. However, under the condition of circle stimulus, the main effect of the type of subject was not significant. These results showed that the P1 amplitudes were significantly greater in the training group than in the non-training group under visual forms of digital stimuli. From the potential period, the main effect of the type of stimulus in the occipital region P1 potential period was significant ($F = 8.538$, $P < 0.01$). The main effect of the type of subject was significant ($F = 15.488$, $P < 0.01$). The P1 potential period of the training group was longer than that of the non-training group, The results indicate that AMC training can prolong the potential period of P1 (**Table 1**).

Comparison in the Central Region N1 Amplitude and the Potential Period Between the Two Groups

The significant effect of forehead central region N1 amplitude was only reflected in the position of the electrode ($F = 23.13$, $P < 0.01$). Analysis of the N1 potential period showed that the main effect of the position of the electrode was significant

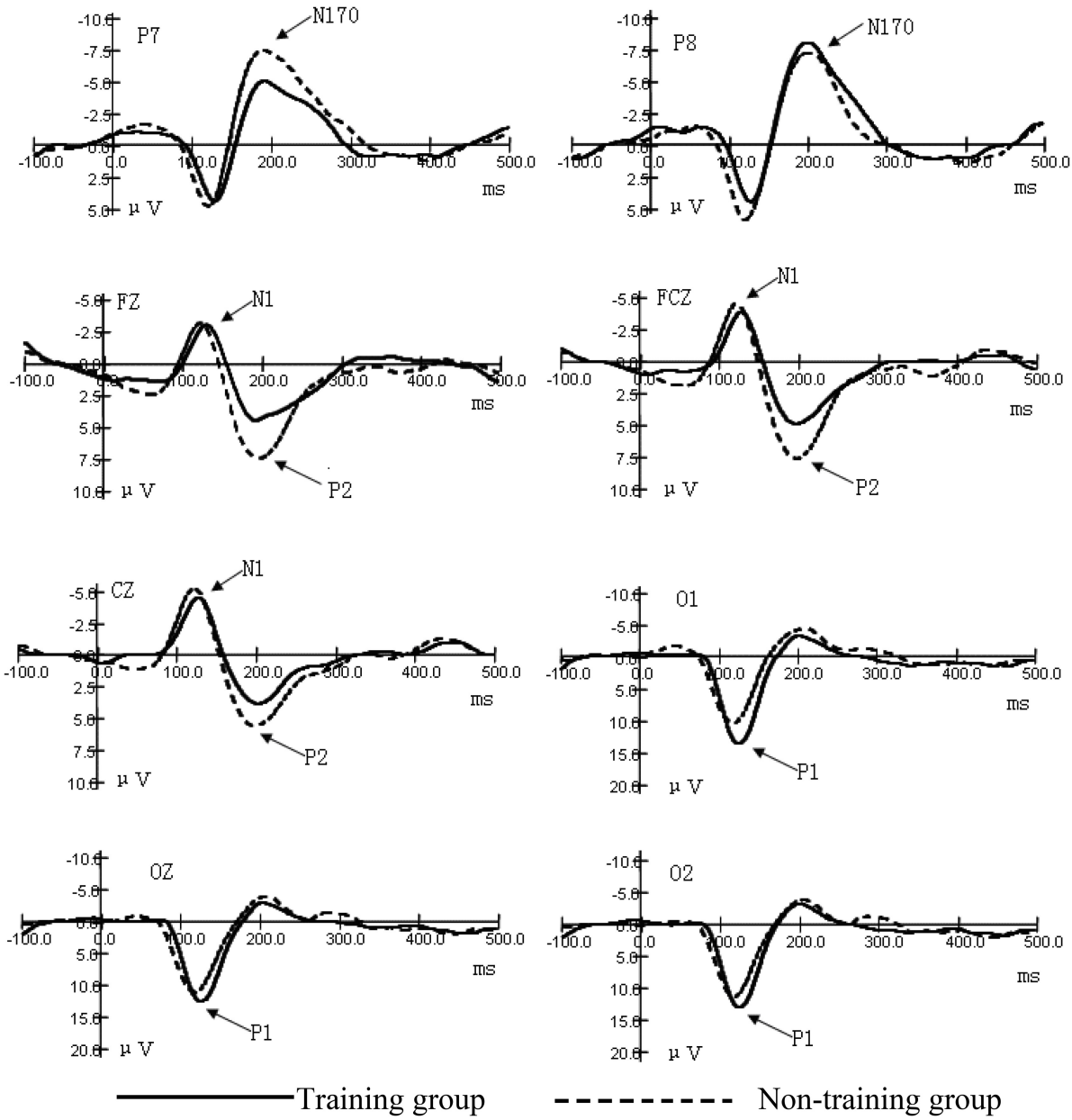

FIGURE 2 | Grand average ERP waveforms for the early components of figures.

($F = 4.72$, $P < 0.05$). The main effect of the type of subject was significant ($F = 13.37$, $P < 0.01$). To sum up, the N1 potential period of the training group was longer than that of the non-training group (**Table 2**).

Comparison of the Temporal Occipital Region N170 Amplitude and the Potential Period Between the Two Groups

The position of the electrode on the temporal occipital region N170 amplitude has a significant main effect ($F = 4.69$, $P < 0.05$). The interaction between the position of the electrode and the type of subject was significant ($F = 3.91$, $P < 0.05$). Further simple analysis showed that in the P7 electrode position, the main effect of the type of subject was significant ($F = 12.98$, $P < 0.01$). The analysis shows that the AMC training has a significant effect on the N170 amplitudes in the P7 position. The AMC training significantly reduced the N170 amplitudes in the temporal regions, especially when stimulated by numbers. The main effect of the type of subject was significant ($F = 4.97$, $P < 0.05$), which shows that, in general, the N170 amplitudes of the training group were smaller than those of the non-training group. The analysis of the N170 potential period showed that the main effect of the subject was near significant ($F = 3.83$,

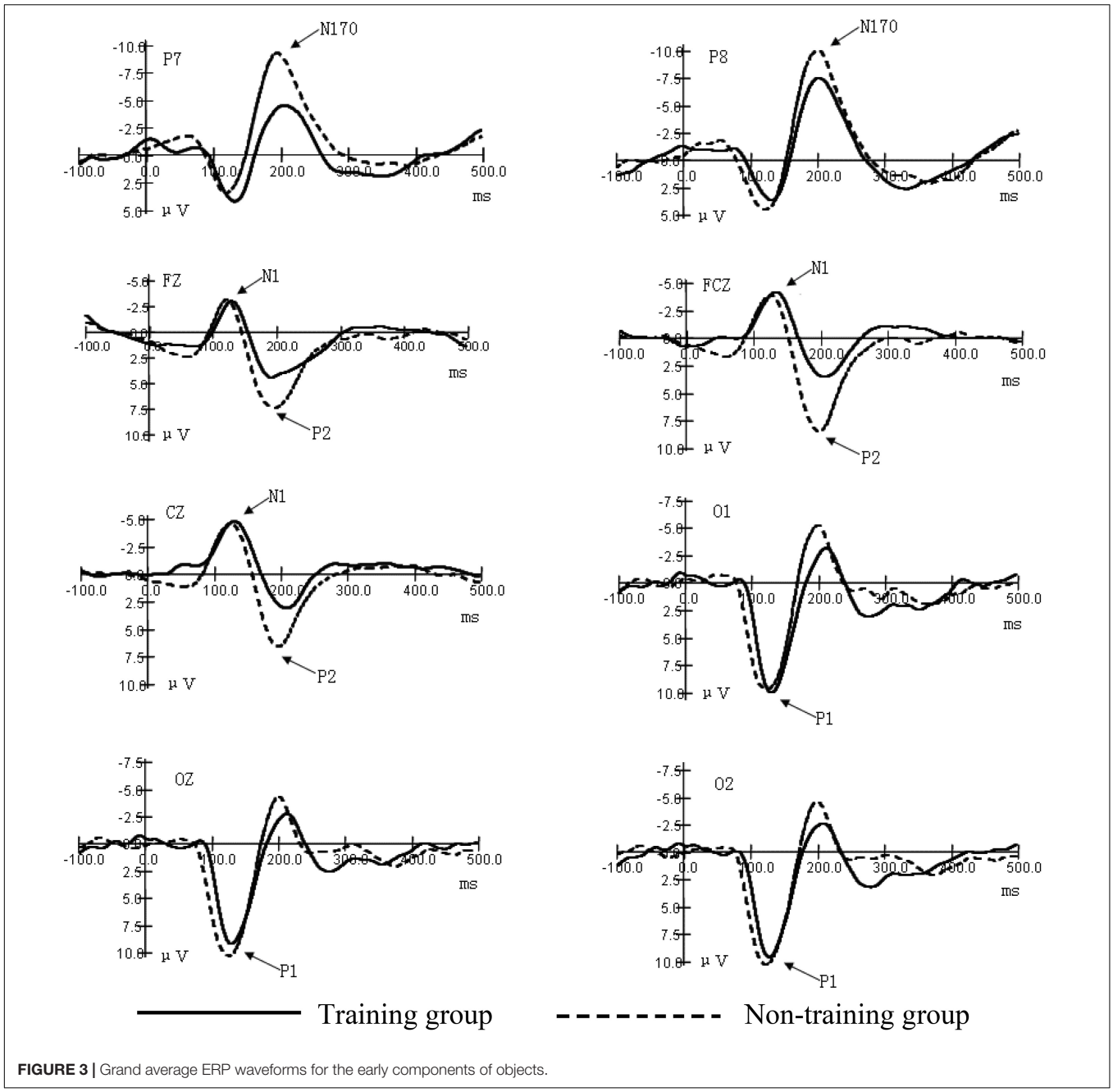

FIGURE 3 | Grand average ERP waveforms for the early components of objects.

P = 0.061). The N170 mean potential period of the training group was longer than that of the non-training group. These suggest that AMC training prolongs the N170 potential period in children (**Table 3**).

Comparison of the Central Region of the Forehead P2 Amplitude and the Potential Period Between the Two Groups

The main effect of the position of the electrode of P2 amplitude was significant (F = 11.05, P < 0.01). The main effect of the type of subject was significant (F = 12.22, P < 0.01). The P2 amplitudes of the training group were smaller than those of the non-training group. The analysis of the potential period showed that the main effect of the type of subject was significant (F = 4.76, P < 0.05). The P2 potential periods of the training group were longer than those of the non-training group. The interaction between the type of stimulus and the type of subject was significant (F = 7.29, P < 0.05). Further simple analysis showed that, under the condition of object stimulus, the main effect of the type of subject was significant (F = 8.41, P < 0.01), which indicates that the AMC training has a significant difference in the amplitudes of P2 induced by the digital stimulus. The P2 potential periods of the training group were longer than those of

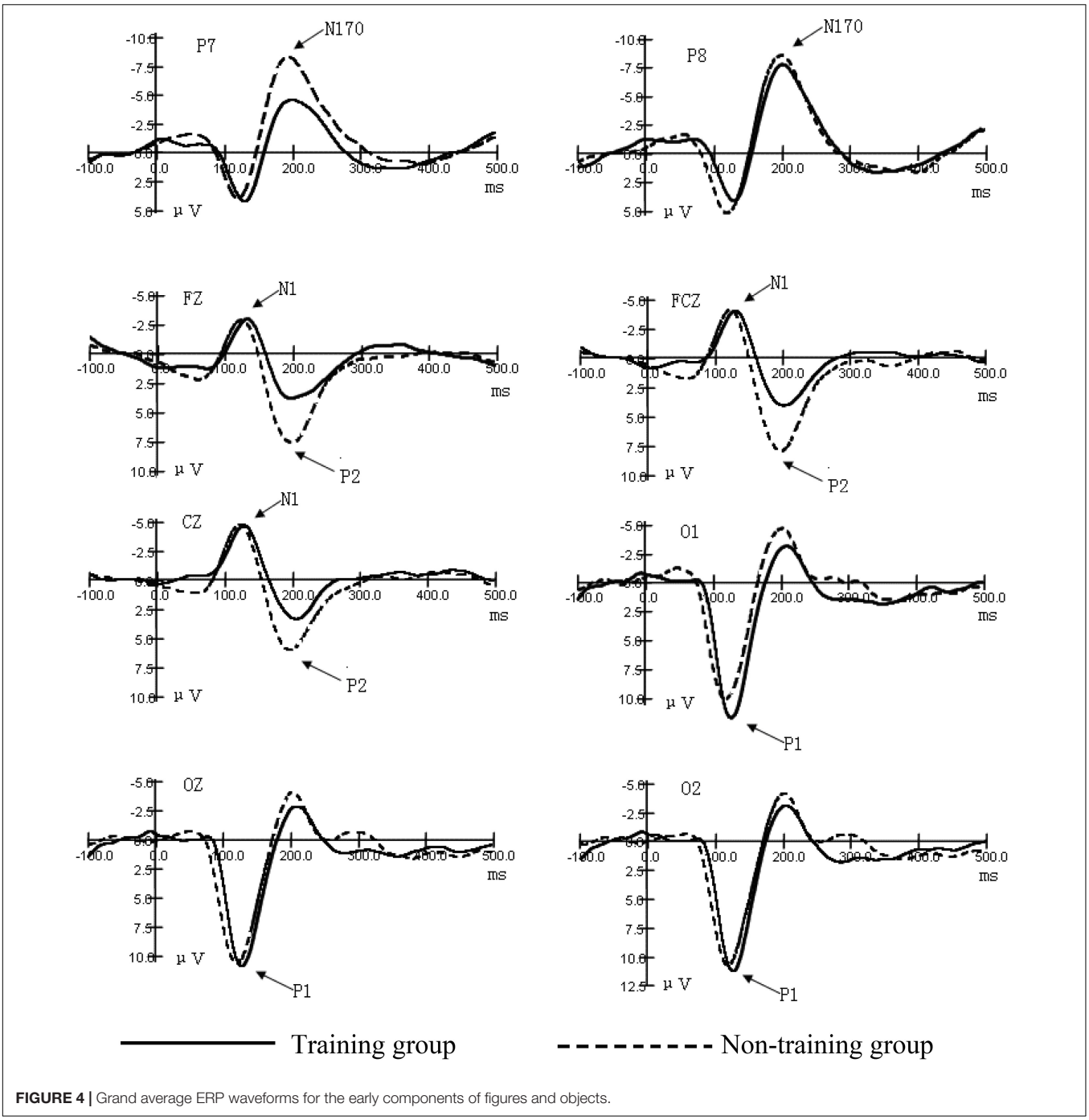

FIGURE 4 | Grand average ERP waveforms for the early components of figures and objects.

the non-training group. Under the condition of figure stimulus, the main effect of the type of subject was not significant (**Table 4**).

DISCUSSION

This is the first study that investigates the effect of AMC on the early process stage of cognition. Our objective was to investigate the effects of AMC training on the early cognitive stage of children. To this end, we used the visual stimulus recognition task, using two different forms of stimulus (number and object), to observe the differences in ERP components between the training and non-training groups. Finally, we compared the amplitudes and potential periods of components. Previous studies have focused on other aspects of cognition, such as working memory, learning ability, mathematical computation, and intelligence, whereas this study is the first to focus on the impact of AMC on early cognitive processing, namely, perception and attention. The results show that a significant difference in the ERP early component of figure perception

TABLE 1 | Comparison of occipital P1 (μV) amplitude and potential period (ms) between the two groups (n = 14, $\bar{x} \pm s$) under the conditions of different stimuli.

Electrode	Group	Object stimulus		Figure stimulus	
		Amplitude (μV)	Potential period (ms)	Amplitude (μV)	Potential period (ms)
O1	Training group	10.90 ± 4.19	132.57 ± 11.69	14.28 ± 4.52	126.43 ± 7.34
	Non-training group	8.50 ± 3.59	124.14 ± 13.42	10.35 ± 3.22	116.5 ± 5.23
OZ	Training group	10.54 ± 4.59	132.50 ± 11.75	13.07 ± 3.67	126.21 ± 7.85
	Non-training group	11.14 ± 3.52	116.78 ± 6.09	10.863.17	116.79 ± 6.10
O2	Training group	10.72 ± 2.83	131.07 ± 12.9	13.62 ± 4.26	127.43 ± 5.83
	Non-training group	10.69 ± 5.01	124.14 ± 10.69	10.83 ± 3.03	117.14 ± 5.08

$\bar{x} \pm s$ (mean ± SD): within the range represents a large probability event and outside the range represents a small probability event.

TABLE 2 | Comparison of forehead central region N1 amplitude (μV) and potential period (ms) between the two groups (n = 14, $\bar{x} \pm s$).

Electrode	Group	Amplitude (μV)	Potential period (ms)
FZ	Training group	3.64 ± 1.59	133.07 ± 8.29
	Non-training group	3.43 ± 3.16	123.07 ± 6.99
FCZ	Training group	4.74 ± 1.89	132.21 ± 7.72
	Non-training group	4.54 ± 3.12	121.54 ± 5.88
CZ	Training group	5.42 ± 2.44	129.25 ± 8.20
	Non-training group	5.17 ± 2.54	121.96 ± 5.46

TABLE 3 | Comparison of the temporal occipital region amplitude (μV) and potential period (ms) between the two groups (n = 14, $\bar{x} \pm s$).

Electrode	Group	Amplitude (μV)	Potential period (ms)
P7	Training group	4.87 ± 3.38	199.64 ± 11.87
	Non-training group	9.57 ± 3.51	189.07 ± 14.18
P8	Training group	7.50 ± 5.01	199.25 ± 8.43
	Non-training group	9.69 ± 5.38	194.67 ± 11.71

TABLE 4 | Comparison of the central region of the forehead P2 amplitude and the potential period between the two groups under different stimulation conditions (n = 14, $\bar{x} \pm s$).

Electrode	Group	Object stimulus		Figure stimulus	
		Amplitude (μV)	Potential period (ms)	Amplitude (μV)	Potential period (ms)
FZ	Training group	4.15 ± 3.58	214.00 ± 16.71	4.95 ± 2.65	207.85 ± 25.31
	Non-training group	8.68 ± 3.95	194.91 ± 10.54	7.80 ± 2.79	201.85 ± 13.23
FCZ	Training group	4.15 ± 3.60	212.71 ± 16.84	5.00 ± 2.79	200.92 ± 18.69
	Non-training group	8.84 ± 3.34	194.42 ± 8.83	8.06 ± 2.30	199.92 ± 12.85
CZ	Training group	3.55 ± 3.15	211.92 ± 16.67	4.00 ± 2.62	207.07 ± 19.95
	Non-training group	6.74 ± 2.76	197.07 ± 16.44	6.67 ± 1.83	206.14 ± 23.55

and circle-object perception exists between the training group and the non-training group and partially proves our hypothesis. The ERP early component reflects the mental process of stimulus discrimination (Simson et al., 1985; Hillyard and Anllo-Vento, 1998). Therefore, this experiment shows that AMC has a significant impact on the early process stage of numbers and objects (particularly numbers), i.e., the sensory perception process stage. The ERP amplitude always reflects the disbursement of the psychological resources as processing information. The amplitude is positively related to the number or strength of neurons activated (Hillyard et al., 1998; Wang et al., 2003). Under the condition of number stimulus, the occipital region P1 amplitudes of the training group are larger than those of the non-training group, which indicates that AMC training enhances the early activation of children to visual information processing. Some studies on brain plasticity show that AMC can enhance the activated degree of some functional regions in the cortex and reorganize the cortex (Hatta and Miyazaki, 1989; Hillyard and Anllo-Vento, 1998). In previous studies, changes in the function of the occipital lobe, parietal lobe, and circuits between these regions were mainly found (Belkacem et al., 2020). We deduce that in brain plasticity, AMC training has a certain influence on the occipital cortex function of children, and this is consistent with previous studies.

Furthermore, this study has found that a significant difference in the N170 component caused by stimulus in the temporal lobes of both sides between the training group and the non-training group exists. N170 is always deemed as the specific component for face recognition (Wang et al., 2019). However, some studies point out that N170 can also reflect visual processing of object discrimination and classification (Rousselet et al., 2004; Caharel and Rossion, 2021). Whether the stimulus is a number or an object, N170 amplitudes of non-mental abacus children are larger than those of mental abacus children, indicating that mental abacus children consume less brain resources than non-mental abacus children as discriminating stimulus.

The component P2 in the central region of the forehead not only reflects the visual coding stage of sensory perception but also relates to the early activation of figure cognitive processing (Kong et al., 1999). In the experiment, P2 amplitudes of the training group are significantly lower than those of the non-training group, indicating that children trained in abacus arithmetic were more likely to have their cognitive processing of numbers activated. The training method of AMC (numbers are converted to imaged beads in the brain) is closely related to

figure processing (Du et al., 2014). Early studies have revealed that number processing is automatic, which means that this process begins immediately and involuntarily upon seeing numbers (Tanaka et al., 2012; Yao et al., 2015), so long-term training may reduce the early activation threshold of children to numbers and improve their automatic degree of cognitive processing of figures. Experiment results show that rear P1, N170, and front N1 and P2 have different potential periods among subjects, indicating that in case of no task, AMC training will extend children's perception time to stimulus even if it can enhance children's early sensory perception and improve attention.

Studies on brain plasticity show that learning and training can change the activation status of different brain areas (Kasahara et al., 2013; Zhou et al., 2020). The findings of this study are consistent with the existing research results.

This study provides a perspective to improve the cognitive ability of children and enhance our understanding of the underlying mechanism of cognitive activity. AMC training can improve children's perceptual ability, and this might be the basis of the superior cognitive processes. We also know that AMC training can alter the activity of some specialized brain regions. Therefore, the introduction of abacus mental arithmetic training in primary education may play a positive role in children's mathematical ability and cognitive development, and pilot education also can be carried out in some regions.

The study also has some limitations. First, the sample only has 28 objects, therefore, the general applicability of conclusions should be considered carefully. However, the experiment is a duplicate measurement trial, and it can make up for the shortage of small samples to some extent. Second, all subjects are children and the average age is only 10.5 years. Notably, cognition is developing. Whether the results that we have observed in children can be embodied in adulthood person is not sure. Finally, we focused only on some aspects of the fundamental capacity of cognition. In the future, we will further investigate the changes in cognitive dimensions associated with mental arithmetic training, while expanding the sample to adults or conducting longitudinal studies.

CONCLUSION

By comparing with ordinary children, the activated degree of the occipital region of the training group is enhanced, while the activated degree of the central region of the forehead and temporal occipital region is slightly down. Meanwhile, the potential periods of all components are extended. Therefore, after long-term AMC training, children's cortical function activities can be improved.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Chaoyang Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DW and KZ were mainly involved in experimental design and testing. JC was mainly involved in testing, data processing, and manuscript writing. JW was mainly involved in manuscript writing. All authors contributed to the article and approved the submitted version.

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Efficacy and Feasibility of an Osteopathic Intervention for Neurocognitive and Behavioral Symptoms Usually Associated With Fetal Alcohol Spectrum Disorder

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The purpose of this study was to evaluate the efficacy and feasibility of a 4-week planned osteopathic manipulative treatment intervention on the improvement of neurocognitive and behavioral symptoms usually associated with fetal alcohol spectrum disorder. Thirty-two symptomatic children without fetal alcohol spectrum disorder aged 3–6 years with low level of attention from two schools and an osteopathic center were recruited in a prospective randomized pilot study in an osteopathic manipulative treatment group [osteopathic manipulative treatment (OMT)] or a control group (standard support measures). Neurocognitive maturity test results for attention (A), iconic memory (IM), spatial structuration (SS), and visual perception (VP) were recorded at baseline and post-intervention. No adverse effects were communicated and there were no dropouts. A significant increase in neurocognitive assessments was observed in children in the OMT group at post-treatment. Intergroup post-intervention statistical differences were found for A, SS, and IM were $p = 0.005$, $p < 0.001$, and $p < 0.001$, respectively; no differences were seen for VP ($p = 0.097$). This study shows that a 4-week osteopathic manipulative treatment intervention may be a feasible and effective therapeutic approach for neurocognitive and behavioral symptoms usually present in fetal alcohol spectrum disorder, justifying more studies on children affected by this condition.

Keywords: fetal alcohol spectrum disorder (FASD), prenatal alcohol exposure, osteopathic manipulative treatment, neurocognitive disorders, attention

INTRODUCTION

Neurocognitive and behavioral symptoms are high incidence disabilities among children with FASD that affect the daily life of the patient, attention deficit being the most frequent (Weyrauch et al., 2017). Several systematic reviews underline the need for further research on the effectiveness of specific interventions aimed at early and individualized treatments of children with fetal alcohol spectrum disorders (FASDs), as well as new effective treatment strategies to improve

neuropsychological symptoms in this population (Reid et al., 2015; Ordenewitz et al., 2021). According to the experience, osteopathy and its application through osteopathic manipulative treatment (OMT) may be an efficient therapeutic tool as an adjuvant treatment in FASDs. A systematic literature review was conducted on using PubMed, Medline and the Cochrane Library with the keywords "Fetal Alcohol Spectrum Disorder," "Fetal Alcohol Syndrome," "Osteopathic Manipulative Treatment," "Neurocognitive Disorders," and "Attention." The reviewed literature indicates that children with FASDs may benefit from interventions when appropriately adapted to their neurodevelopmental disabilities (Petrenko, 2015) and may help improve their health-related quality of life (Stade et al., 2006). Similarly, there should be acceptance of the interventions by the patients and their families (Petrenko, 2015). This is very relevant for sensitive populations, such as the families with FASD members (Domeij et al., 2018; Flannigan et al., 2020; McLachlan et al., 2020; Pruner et al., 2020), particularly in communities such as ours, were prevalence of FASD of internationally adopted children is very high (Catalunya, 2019; Palacios et al., 2019).

Despite the global increase in the practice and specialization of pediatric osteopathy (International Alliance, 2020; DeMarsh et al., 2021; Schwerla et al., 2021), and its low-risk-profile (Hayes and Bezilla, 2006; DeMarsh et al., 2021), further research is needed to gather a body of evidence that could be used to recommend pediatric OMT under specific clinical conditions (DeMarsh et al., 2021). Thus, following recent recommendations in the literature (DeMarsh et al., 2021), assessment of viability and safety of OMT interventions in pediatric osteopathy are required before their use on specific population groups.

This preliminary study was designed on the assumption that the FASD population and their families will be receptive to experimental interventions (Stade et al., 2006; Domeij et al., 2018) and because of the lack of studies assessing the efficacy of OMTs on FASD-related neurocognitive and behavioral symptoms (Reid et al., 2015; Petrenko and Alto, 2017; Ordenewitz et al., 2021).

Positive effects of therapeutic interventions on neuropsychological symptoms in people with FASD have been shown, indicating that gains on attention (A) may be achieved, and generalize to other areas of functioning (Reid et al., 2015; Petrenko and Alto, 2017; Ordenewitz et al., 2021).

One of the purposes of osteopathy is to detect and correct somatic dysfunctions and their potential negative effects through manual contact by OMT. The results of research carried out to date suggest that OMT has anti-inflammatory (Standley and Meltzer, 2008; Licciardone et al., 2012; Degenhardt et al., 2017) and parasympathetic effects (Henley et al., 2008; Giles et al., 2013; Ruffini et al., 2015). Although specific metabolic and neurological alterations linked to the somatic dysfunction have been identified (Van Buskirk, 1990; Korr, 1991; Snider et al., 2011), the underlying physiological mechanisms remain under study (Tozzi, 2015; Tramontano et al., 2020; Roura et al., 2021). Moreover, there is evidence on the relation between the somatosensory system and neurological development processes, particularly in the areas of perception and cognition. Recent research has shown a dynamic interaction between the somatosensory system and a-related brain centers (Dockstader et al., 2010; Haegens et al., 2012; Wiesman and Wilson, 2020). Furthermore, other works have demonstrated effects on cortical plasticity after OMT interventions (Ponzo et al., 2018), as well as specific brain connectivity changes in sensorimotor, locomotor, and postural function networks, which suggests an alteration in the processing of information post-OMT (Tramontano et al., 2020).

Over the past years, there has been an increase in the number of publications on pediatric OMT, with additional evidence of its benefits in the field of neurological development disorders (DeMarsh et al., 2021). Nevertheless, further research is needed on the effectiveness of OMT in children (Parnell Prevost et al., 2019; DeMarsh et al., 2021).

Improvement of A in children and adolescents with attention deficit hyperactivity disorder (ADHD) has been seen (Accorsi et al., 2014), as well as positive effects in learning processes and infant neurological development (Frymann, 1976; Frymann et al., 1992). Social behavior and communication indexes ameliorated in a sample of children with autism (Bramati-Castellarin et al., 2016) as well as the mood, sleep, and limb function in children with cerebral palsy (Duncan et al., 2004). There is convincing evidence on the positive effect of OMT as adjuvant treatment in premature infants in neonate intensive care units (ICUs), e.g., decreased hospital stays and associated costs (Lanaro et al., 2017). Therefore, interventions such as an OMT may aid in neurocognitive and behavioral pediatric development including those to FASDs.

We hypothesized that standardized OMT aimed to correct individualized somatic dysfunctions would improve measures indicated by the Cumanin® measuring test. The main primary objective of this pilot study was to evaluate the efficacy and feasibility of a 4-week planned OMT intervention delivered by a qualified pediatric osteopath, on Attention (A), iconic memory (IM), spatial structuration (SS) and visual perception (VP) in a group of children without FASD with low levels of A. The main secondary objective was to validate the intervention to apply it to FASD population in future studies.

MATERIALS AND METHODS

Study Design

Prospective randomized pilot study.

Patients

Children aged 3 to 6 years without a FASD diagnosis but with symptoms usually present in FASDs (Kodituwakku, 2009; Lange et al., 2017; Weyrauch et al., 2017; Maya-Enero et al., 2021) identified through a neuropsychological assessment referred from schools and an osteopathic center, were recruited between June 1 and July 17, 2020. Children with A and behavior problems according to their parents and/or teachers, following inattention criteria in the DSM-5 handbook were pre-selected (Battle, 2013). Reduced levels of A were recognized using the Neuropsychological Maturity Questionnaire for Children (Cumanin®) (Portellano Pérez et al., 2009) before the intervention during the recruitment process.

Decreased levels of A were considered with scorings below the 50th percentile ($p > 50$) in the attention scale (Portellano Pérez et al., 2009). Due to the absence of previous studies, it was not possible to perform the estimated calculation of the sample size for this pilot study.

Children diagnosed with ADHD, or other neurological, genetic, and/or metabolic pathology, or receiving pharmacological treatment at the beginning of the intervention or had undergone OMT over the 12 months prior to the intervention, were excluded.

Informed consent to participate in the study was obtained from the parents/legal tutors, who also received written and verbal information on the design of the study and protocol. The Ethical Committee for Clinical Research Parc de Salut MAR (Barcelona, Spain) approved the study protocol (2016/7052/I), conducted according to the guidelines of the Declaration of Helsinki for Human Research of 1964 (last modified in 2013).

Interventions

Two groups of children were defined: the OMT group ($n = 16$) children who received three OMT sessions over a 4-week period (one session every 2 weeks). Permuted-block randomization was used for treatment allocation. A research associate generated the random sequence using the Excel software. The control group ($n = 16$) were children who received standard support measures. Participants from both groups got the same tailored standard support learning measures at their schools, following the standard guidelines of educational intervention based on the creation of enabling environments and individualized support adapted to children with neurocognitive and behavioral symptoms, e.g., low level of A (Battle, 2013; Catalunya, 2019). Support measures received by the participants at school throughout the study period were not modified.

At the first intervention, each participant underwent a protocolized anamnesis and an osteopathic physical examination based on SOAP (Subjective, Objective, Plan, Assessment) notes and exam forms (Sleszynski et al., 1999; Sleszynski and Glonek, 2005). Somatic dysfunctions were detected by physical examination, based on tissue texture changes, asymmetry, limitation in normal range of motion, and tissue tenderness parameters (TART), which guided the osteopathic evaluation and OMT intervention. The parameters of somatic dysfunctions were described by the position and motion of a body part as determined by palpation.

Using OMT techniques, the identified somatic dysfunctions were corrected one by one in the whole body (Tramontano et al., 2020). The following approaches were used: balanced ligamentous techniques, balanced membranous techniques/osteopathy in the cranial field, and facilitated positional release techniques (Johnson and Kurtz, 2003). An osteopathic physical examination and an OMT intervention were performed in each session to assess and correct somatic dysfunctions. The time allocated for the first session was 50 min, and the next two 30 min each.

To improve adherence and reduce performance bias, participants were assigned the OMT the same day every week. Reminder and confirmation calls were made to families 24 h before each scheduled intervention and before the pre-/post-tests.

A qualified pediatric osteopath, with a master's degree in Osteopathy, following the recommendations of the European Standard UNE-EN 16686 (16686:2015), and a postgraduate specialization in Pediatric Osteopathy, carried out the OMT interventions. A qualified psychologist performed the neuropsychological pre-/post-tests to all participants at baseline and at conclusion of the intervention (the day after the last OMT session). Specific and general recommendations of each questionnaire were followed. Pre- and post-tests took between 20 and 30 min each (Portellano Pérez et al., 2009).

Outcome Variables

The primary outcome for validating and assessing the OMT was the percentage of patients who completed the intervention and showed statistically significant differences in the individually administered Cumanin® measuring test, which includes neuropsychological maturity scales that allow to determine the centile values for A, IM, SS, and VP (Portellano Pérez et al., 2009). Investigators who performed and assessed the OMT were blinded to patient random allocation.

Description and Neurofunctional Significance of the Neurocognitive Scales

Attention (A): 20 items – the aim was to identify and mark 20 geometrical figures identical to the proposed model (a square) shown among 100 figures, 80 of which were distractors and 20 squares identical to the model. The test was carried out for 30 sec and the correct answers (correctly crossed-out squares) and errors (other incorrectly crossed-out figures) were noted, although only the number of correctly crossed-out figures was taken into account. Maximum score = 20; minimum = 0. This assesses structures that are involved with A processes, particularly reticular formation and prefrontal cortex. The right cerebral hemisphere is dominant in A control (Portellano Pérez et al., 2009).

Iconic memory (IM): 10 items – the child had to memorize 10 simple drawings of objects for 1 min. Then, the child had to say the name of the drawings he remembered, in a period of 90 sec. The child got 1 point for each well-remembered object. It was not considered if child said an incorrect object. Maximum score = 10; minimum = 0. Immediate memory is related to structures such as the hippocampus, parietal cortex, and amygdala. This scale evaluates right hemisphere function (Portellano Pérez et al., 2009).

Spatial structuration (SS): 15 items – the child had to perform increasingly difficult spatial orientation activities via psychomotor (11 items) and graphomotor responses (4 items). Maximum score = 15; minimum = 0). Essentially, this is related with association centers at the parietal-temporal-occipital cortex, in charge of spatial representation on the Penfield sensory homunculus at the parietal cortex (Portellano Pérez et al., 2009).

Visual perception (VP): the child had to reproduce 15 items geometrical designs of increasing difficulty. Each correctly drawn figure was valued with 1 point. The test ended if the child made 4 consecutive drawings wrong. Maximum score = 15; minimum = 0. Secondary visual areas and associative areas on the occipital lobe mediate this, as well as the mnemonic function, which is mediated by deeper

areas of the temporal cortex. The frontal cortex is also involved, along with various motor-decision centers of the brain (Portellano Pérez et al., 2009).

Each scale allows scores to be recorded, the interpretation of which is made by converting these raw scores into centile scales, which are differentiated into five age groups in months. Scores below normal are considered to be centiles from 20 to 40, with scores below the 20th centile being considered very low (Portellano Pérez et al., 2009).

Statistical Analysis

All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) v.21.0 for Windows (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, United States: IBM Corp). Categorical variables were evaluated using frequencies and percentages and quantitative variables with means and standard deviations that included maximum and minimum values (range). Distribution of the data was evaluated using Shapiro-Wilk test. Comparisons at various time intervals within each group were analyzed using Friedman's test and, if statistical significance was detected, multiple comparisons were carried out using Wilcoxon's sign rank test. Categorical variables were analyzed using the Pearson's chi-square (χ^2) test. Groups were compared with Kruskal-Wallis test complemented by the Bonferroni correction. Cohen's d was calculated to evaluate effect sizes. *P* values < 0.05 were considered statistically significant.

RESULTS

Thirty-two participants ($n = 32$) without FASD were included in this study, 16 in the OMT group and 16 in the control group. Gender ratio (male: female) was 17:15; 10:6 for the OMT group and 7:9 for control group. No adverse effects were communicated and none of the participants dropped out. Demographic characteristics of children from both groups are shown in **Table 1**.

Average duration of the interventions was as follows: anamnesis -1st session- 16.25 min, exploration, 10.83 min, and treatment, 10.42 min. Average number of somatic dysfunctions (SD) were [most prevalent: cranial (30.0%), diaphragm (17.1%), and cervical area (12.8%)] per participant at baseline was 4, dropping to 1.5 at the last OMT session. Percentages of the used approaches were as follows: balanced ligamentous techniques (61.4%), balanced membranous techniques/osteopathy in the cranial field (30%), and facilitated positional release (8.6%).

Participant Flow

Forty-three ($n = 43$) children were pre-selected; eight were excluded because of a percentile above 50 in the A scale. Thirty-five candidates ($n = 35$) were enrolled, of whom three were excluded for not meeting the inclusion criteria, i.e., had received OMT treatment over the past 12 months ($n = 2$) and undergoing pharmacological treatment ($n = 1$). Thirty-two participants were finally included in the study, 16 randomly allocated to the OMT group and 16 to the control group. One osteopath from a single osteopathic center delivered the OMTs. Standard support measures were applied at school ($n = 12$). Statistical analyses were performed including the 32 participants. All completed the treatment and there were no dropouts.

Statistically significant differences were observed for A in the control group ($p = 0.027$) and in the OMT group ($p = 0.031$) (**Table 2**) following Friedman's test; similarly, differences ($p < 0.001$) were found for SS in the control and OMT groups (**Table 3**). Statistically significant differences were seen for VP only in OMT group ($p = 0.019$) (**Table 4**); no statistically significant pre-post results were seen for IM in the control group, contrary to what was observed in the treatment group ($p < 0.001$) (**Table 5**).

Post-treatment statistically significant differences were found for A, SS, and IM between the treatment and control groups ($p = 0.005$, $p < 0.001$, and $p < 0.001$, respectively). This was not the case for VP, for which no statistical differences ($p = 0.097$)

TABLE 1 | Baseline characteristics of the study groups.

	Control group ($n = 16$)	OMT group ($n = 16$)	*P* value
Boys/girls	7/9	10/6	0.288
Age at study entry (months), mean (SD)	48.81 (10.001)	54.75 (9.370)	0.093
Attention, median [IQR]	27.50 [15.00–35.00]	20.00 [5.00–35.00]	0.152
Iconic memory, median [IQR]	75.00 [61.25–80.00]	75.00 [60.00–80.00]	0.931
Spatial structuration, median [IQR]	65.00 [60.00–70.00]	80.00 [35.00–93.75]	0.085
Visual perception, median [IQR]	45.00 [40.00–75.00]	57.50 [40.00–78.75]	0.212

IQR, interquartile range; OMT, osteopathic manipulative treatment; SD, standard deviation.

TABLE 2 | Neuropsychological maturity test average centile scores for attention.

	Attention			
	Pre-intervention, median [IQR]	Post-intervention, median [IQR]	Effect size (within group)	*P* value
Control	27.50 [15.00–35.00]	17.50 [15.00–23.75]	−0.58	0.027
OMT	20.00 [5.00–35.00]	30.00 [20.00–43.75]	0.34	0.031

IQR, interquartile range; OMT, osteopathic manipulative treatment.

TABLE 3 | Neuropsychological maturity test average centile scores for spatial structuration.

	Spatial structuration			
	Pre-intervention, median [IQR]	Post-intervention, median [IQR]	Effect size (within group)	*P* value
Control	65.00 [60.00–70.00]	30.00 [21.25–50.00]	−1.80	<0.001
OMT	80.00 [35.00–93.75]	57.50 [30.00–83.75]	−0.68	<0.001

IQR, interquartile range; OMT, osteopathic manipulative treatment.

TABLE 4 | Neuropsychological maturity test average centile scores for visual perception.

	Visual perception			
	Pre-intervention, median [IQR]	**Post-intervention, median [IQR]**	**Effect size (within group)**	***P* value**
Control	45.00 [40.00–75.00]	45.00 [26.25–48.75]	−0.31	0.184
OMT	57.50 [40.00–78.75]	47.50 [35.00–70.00]	−0.42	0.019

IQR, interquartile range; OMT, osteopathic manipulative treatment.

TABLE 5 | Neuropsychological maturity test average centile scores for iconic memory.

	Iconic memory			
	Pre-intervention, median [IQR]	**Post-intervention, median [IQR]**	**Effect size (within group)**	***P* value**
Control	75.00 [61.25–80.00]	65.00 [60.00–80.00]	−0.32	0.117
OMT	75.00 [60.00–80.00]	90.00 [80.00–95.00]	1.22	<0.001

IQR, interquartile range; OMT, osteopathic manipulative treatment.

were determined by Kruskal-Wallis test complemented by the Bonferroni correction (**Table 6**).

No relevant adverse events or side effects were communicated.

DISCUSSION

The objective of this study was to evaluate the efficacy and feasibility of an OMT intervention on neurocognitive and behavioral symptoms commonly present in FASD (Kodituwakku, 2009; Weyrauch et al., 2017; Maya-Enero et al., 2021), and validate the intervention to apply it to FASD population in the future.

This work shows that a 4-week OMT plan, administered by a qualified pediatric osteopath, is a feasible therapeutic approach for children aged 3–6 years who exhibit neurocognitive and behavioral symptoms usually present in FASD, such as attention deficit (Kodituwakku, 2009; Lange et al., 2017; Weyrauch et al., 2017; Maya-Enero et al., 2021), effectively improving A, SS, and IM, but not VP.

The development of perception and cognition is linked to the somatosensory system (Dockstader et al., 2010; Haegens et al., 2012; Wiesman and Wilson, 2020) and the potential effects of somatic dysfunctions (Frymann, 1976; Frymann et al., 1992; Accorsi et al., 2014). The sociodemographic profile of the FASD population in our community (Catalunya, 2019) is characterized by a high index of FASD children who have been adopted from countries of Eastern Europe, with high prevalence of special needs (Palacios et al., 2019) and may thus be susceptible to experimental interventions. Thus, in our opinion, prior validation of any new therapeutic intervention aimed at this population should be a priority. Therefore, the current study evaluates cases with neurocognitive and behavioral symptomatology without a FASD diagnosis (Maya-Enero et al., 2021). The aim was to assess the feasibility of an OMT intervention that could be used for FASD individuals. Our results, as a preliminary intervention tool, show that OMT can be a valid approach for treating neurocognitive and behavioral symptoms usually present in FASDs (Kodituwakku, 2009; Lange et al., 2017; Weyrauch et al., 2017; Maya-Enero et al., 2021).

Research evidence indicates that gains in A can be achieved in FASD populations (Reid et al., 2015; Petrenko and Alto, 2017; Ordenewitz et al., 2021).

In our review of the literature we did not find studies evaluating OMT interventions on cases with neurocognitive and behavioral symptoms usually associated with FASD (Reid et al., 2015; Petrenko and Alto, 2017; Ordenewitz et al., 2021). Moreover, there is lack of relevant studies measuring the efficacy of OMT on neuropsychological development. Accorsi et al. suggest that OMT may improve selective and sustained A performances in children and adolescents with ADHD (Accorsi et al., 2014), although this should be further investigated.

Absence of adverse effects in our study may be due to the lower incidence of adverse events immediately after the OMT in comparison to other manual medical disciplines (Degenhardt et al., 2018), while the gentle, non-invasive, tailored health care approach of OMT may have helped maintain patient adherence (World Health Organization, 2012). Possibly, protocolized osteopathic anamnesis and examination, the training and experience of the care providers, and supervision of the procedures, are additional factors that may have contributed to the success of the interventions. Families of children affected by FASD show great interest in receiving care and treatment (Lange et al., 2018; Flannigan et al., 2020), which may help maintain a low dropout rate in future interventions with this population. Further research is required to assess OMT efficacy and patient's safety (Degenhardt et al., 2018).

In this study, we show positive post-OMT outcomes on a defined population, significant in three of the four assessed variables. These results may be because OMT interventions

TABLE 6 | Post-treatment average score differences in the two study groups.

	OMT group, median difference [IQR]	**Control group, median difference [IQR]**	**Effect size (between group)**	***P* value**
Attention	10.00 [7.75–15.00]	−10.00 [−11.25–0.00]	0.62	0.005
Iconic memory	15.00 [15.00–20.00]	−10.00 [−13.00- −4.00]	0.86	<0.001
Spatial structuration	−22.50 [−29.00- −5.00]	−35.00 [−38.75- −20.00]	0.79	<0.001
Visual perception	−10.00 [−18.75- −5.00]	0.00 [−7.00–5.50]	−0.51	0.097

IQR, interquartile range; OMT, osteopathic manipulative treatment.

have on somatic dysfunctions, which consequently reduce the potential negative consequences on perceptual and cognitive development (Frymann, 1976; Frymann et al., 1992; Tozzi, 2015). More research is needed to assess the effect of OMT interventions in children younger than 6 years with low levels of A. Post-treatment results show a favorable effect of overall neuropsychological development OMTs toward the negative evolution of these variables over time. Although the characteristics of this study do not allow to draw additional conclusions, the results suggest the need of more in-depth studies on the evolution of overall neuropsychological development in pre-school children as stated by Sjöwall et al. (2017) study. However, early neuropsychological deficits may be identified and have predictive value in future development of ADHD symptoms and subsequent academic performance (Sjöwall et al., 2017). Still, the small sample size and duration of the study limit any conclusion. More studies with larger samples and longer study duration are recommended.

The average number of somatic dysfunctions per participant at baseline was 4 [most prevalent: cranial (30%), diaphragm (17.1%), and cervical area (12.8%)] and 1.5 at the last session. OMT interventions may explain the observed positive results on somatic dysfunctions. These results seem to corroborate data from previous works (Accorsi et al., 2014), although the characteristics of our study limit further comparisons. The different levels of improvement may be explained by the various development processes and maturation pathways of each measured variable (Portellano Pérez et al., 2009), suggesting that somatic dysfunctions and OMT interventions may have distinct effects on each process. Additional research is needed to deepen into the mechanisms of OMT on somatic dysfunctions (Tozzi, 2015). Brain plasticity and neurodevelopment mechanisms present during the first stages of life may explain the positive effects observed in our work despite the short duration of the study (Portellano Pérez et al., 2009; Lange et al., 2017). This supports the importance of early interventions in neurocognitive and behavioral disorders (Portellano Pérez et al., 2009; Reid et al., 2015; Petrenko and Alto, 2017; Ordenewitz et al., 2021).

Despite the relevant findings, this study has some limitations. This is a pilot study showing a favorable effect of OMT on children between 3 and 6 years of age with attention deficits. More research is needed to assess whether this intervention may be able to help all children with attention problems, including those with FASD. Attention deficit in our study population may have a different etiology than that of the FASD population, which may lead to distinct post-intervention results and conclusions in comparison to those observed in a FASD population (Glass et al., 2013; Boseck et al., 2015). In cases of PAE, the impact of combined genetic and epigenetic factors throughout pre- and postnatal development, makes it difficult to establish a specific neuropsychological profile (Mattson et al., 2019; Maya-Enero et al., 2021) or determine its progression over time (Weyrauch et al., 2017).

Other limitations are the small sample size, which restricts the assessment of efficacy, and age of participants. In the latter, OMT on individuals aged 3 to 6 years would enable to intervene in early neural development and deliver the intervention during early neurodevelopmental difficulties. However, the number and variety of neurocognitive and behavioral evaluation tools for children under 6 years is scarce (Portellano Pérez et al., 2009; Coles et al., 2021). The short length of the study is a limitation to objectify improvements in neuropsychological development. Moreover, the capacity children have for learning and remembering the tests may be a bias in terms of evaluation (Portellano Pérez et al., 2009). To homogenize our sample based on attention deficit, we carried out an assessment of A using the scale of the Cumanin® neuropsychological battery throughout 4 weeks before the intervention, a factor that may increase the recall bias in the variable. The above-mentioned limitations can be reduced by increasing the size of the sample and study duration, as well as an extended follow-up period beyond the post-treatment period (Reid et al., 2015), as this would allow to determine if the achieved results are maintained over time. Moreover, other assessment tools can be used before the intervention during the recruitment period to reduce the recall bias for this variable.

Cumanin® is a neuropsychological assessment instrument validated in Spain for children between 36 and 78 months of age, widely used in Spain and other Spanish speaking countries (Urzúa et al., 2010; Ávila Matamoros, 2012; Salvador-Cruz et al., 2019). This means that the results may be not reproducible in samples from populations from different countries. The reliability of the questionnaire is considered acceptable and supported by a study that includes a sample of 803 participants (Portellano Pérez et al., 2009). Thus, four specific scales of the Cumanin® questionnaire were used. Although the scales have been designed to measure each variable independently, using the scales separately may imply a potential bias. This was compensated by strictly following the instructions and steps described for the evaluation (Portellano Pérez et al., 2009). During the drafting of this manuscript, a new version of the Cumanin questionnaire was published (Cumanin®-2) (Portellano-Pérez et al., 2021), an extended and updated version of Cumanin® for the neuropsychological assessment of children. Our study did not aim to assess and analyze the overall neuropsychological status of the participants, but to evaluate the selected variables and determine their evolution over time. Therefore, the used assessment tools in this work retain their validity regarding pre- and post-intervention assessments. Moreover, in future interventions involving FASD populations within this age range, the use of Cumanin®-2 should be considered, because to date, there is no references in the literature that describe the clinical significance of the changes in the measurements of the scales used.

Participants and their families were not blinded to the OMT intervention, and no sham-intervention or placebo treatment was offered due to the lack of standard guidelines for OMT use (Cerritelli et al., 2016). Therefore, a placebo effect should be considered in the current study, which can be overcome by a homogeneous well-reported sham therapy applied to a third group, using a wait-listed control group or a crossover study design in future studies. Due to its importance, the creation of standard well-reported placebo treatments and their application in OMT clinical trials should be considered in further works (Cerritelli et al., 2016). In addition, the lack of a predetermined

treatment protocol limits the generalizability of the results. Moreover, this factor allows the intervention on the FASD population to be tailored to the patient's profile and symptoms, as noted in a recent systematic review (Ordenewitz et al., 2021). This is a common obstacle in the field of manual medicine that can be minimized by applying standardized procedures (Alvarez et al., 2016). Following anamnesis and exploration protocols, discussion and supervision of the procedures among several professionals, and specific training and education of care providers, were measures adopted to minimize this limitation.

Although the characteristics of this study do not allow drawing further conclusions, our results suggest the need for further studies on certain clinical presentations characterized by deficits in neurocognitive and behavioral development, a field explored by Frymann et al. (1992) several years ago (Frymann, 1976).

CONCLUSION

Our study provides important data supporting the need for more rigorous trials. Statistically significant post-intervention differences between treatment and control groups were observed for A, SS, and IM; no differences were seen for VP. Significative changes in A and IM were observed in the treatment group. No adverse effects were communicated and none of the participants dropped out. Our results justify the design of a controlled clinical study to evaluate the feasibility and efficacy of OMT interventions in FASD populations with larger samples, extended follow-up periods, and a sham therapy to a third group of participants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee for Clinical Research Parc de Salut Mar, Barcelona, Spain (2016/7052/I). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RC-S conceived the experiments. RC-S, MA-V, and ÓG-A designed and performed the experiments. DV-D analyzed the data. RC-S, DV-D, MA-V, and ÓG-A wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Sex Differences in Demographic and Pharmacological Factors in Alzheimer Patients With Dementia and Cognitive Impairments

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Objective: The current study investigates sex differences associated with pharmacological and demographic characteristics in Alzheimer patients (AD) with dementia (ADD) or mild cognitive impairment (MCI).

Method: A retrospective analytical approach was used to analyze data from 45,696 AD patients with MCI or ADD. The univariate analysis was used to determine differences in demographic, and pharmacological characteristics for male and female ADD and MCI-AD patients. Multivariate analysis was used to predict specific pharmacological and demographic factors that are associated with male and female MCI and ADD patients.

Result: In the adjusted analysis for male patients, Hispanics [0.166,0.020 – 1.355, $P = 0.094$] or African Americans [OR = 2.380, 95% CI,2.120 – 2.674, $P < 0.001$], were more likely to have MCI-AD and be treated with galantamine [OR = 0.559, 95% CI, 0.382 – 0.818, $P = 0.003$], donepezil [OR = 1.639, 95% CI,1.503 – 1.787, $P < 0.001$], rivastigmine [OR = 1.394, 95% CI,1.184 – 1.642, $P < 0.001$], olanzapine [OR = 2.727, 95% CI,2.315 – 3.212, $P < 0.001$], risperidone [OR = 2.973, 95% CI,2.506 – 3.526, $P < 0.001$], present with increasing age [1.075,1.071 – 1.079, $P < 0.001$], and are on tobacco use [OR = 1.150, 95% CI,1.054 – 1.254, $P = 0.002$]. For female patients, buspirone [OR = 0.767, 95% CI, 0.683 – 0.861, $P < 0.001$] and a history of alcohol (ETOH) use [OR = 0.484, 95% CI, 0.442 – 0.529, $P < 0.001$] were associated with MCI-AD. Increasing age [OR = 1.096, 95% CI, 1.093 – 1.100, $P < 0.001$], donepezil [OR = 2.185, 95% CI, 2.035 – 2.346, $P < 0.001$], memantine [OR = 2.283, 95% CI, 2.104 – 2.477, $P < 0.001$] aripiprazole [OR = 1.807, 95% CI, 1.544 – 2.113, $P < 0.001$] olanzapine [OR = 2.289, 95% CI, 1.986 – 2.640, $P < 0.001$] risperidone [OR = 2.548, 95% CI, 2.246 – 2.889, $P < 0.001$] buspirone [OR = 0.767, 95% CI, 0.683 – 0.861, $P < 0.001$] escitalopram [OR = 1.213, 95% CI,1.119 – 1.315, $P < 0.001$] African Americans [OR = 1.395, 95% CI, 1.268 – 1.535, $P < 0.001$] and tobacco use [OR = 1.150, 95% CI, 1.073 – 1.233, $P < 0.001$] were associated with ADD.

Conclusion: Our findings reveal that MCI-AD patients were more likely to be Hispanics or African American males treated with rivastigmine, olanzapine and citalopram. African American females were associated with ADD and more likely to be treated with buspirone and presented with a history of ETOH. This finding suggests the need for a pharmacological treatment approach encompassing sex-sensitive strategies for MCI-AD and ADD patients.

Keywords: gender -, demography, Alzheimer's Disease, dementia, cognitive impairment

INTRODUCTION

In the elderly, AD is a commonly observed etiology of mild cognitive impairment (MCI) and early dementia (Lu et al., 2021), and both are characterized by cognitive impairment (Petersen, 2016). The significant difference between MCI and dementia is that in dementia, more than one cognitive domain is affected, resulting in interference in activities of daily living (Knopman and Petersen, 2014). The prognosis for MCI and dementia is an essential motivation for early accurate diagnosis, as in both, there is a risk for further cognitive decline. In persons over age 70 years, more than 13% are reported to present significant cognitive impairment to warrant a diagnosis of dementia (Knopman and Petersen, 2014). Although a diagnosis of MCI may be made and later rescinded because of improvement in cognition, once diagnosed with MCI, individuals are at greater risk for future decline than those who never had MCI (Knopman and Petersen, 2014; Langa and Levine, 2014). In contrast, persons with dementia almost invariably worsen over time (Petersen et al., 2010). In MCI associated with AD (MCI-AD) and dementia associated with AD (ADD), cognitive function is characterized by the Diagnostic and Statistical Manual of Mental Disorders (DSMMD) into 5 domains: (1) learning and memory, (2) language, (3) visuo-spatial, (4) executive, and (5) psychomotor (Tarawneh and Holtzman, 2012; Knopman and Petersen, 2014). For a diagnosis of MCI, only one of these domains must be impaired in order to make a diagnosis, whereas more than one domain are impaired to make a diagnosis of dementia (Tarawneh and Holtzman, 2012).

Females with MCI are reported to present with greater longitudinal rates of cognitive and functional decline than males (Tarawneh and Holtzman, 2012), and females make up almost two-thirds of AD patients in the United States (Hebert et al., 2013). The explanation provided for the higher cases of AD and rates of cognitive impairments in females is often linked to their greater longevity, and sociocultural factors (Mielke et al., 2014). The existing literature is far from conclusive and consists mainly of hypotheses that involve sex-specific biological and sex-specific sociocultural factors that increase females' vulnerability over males (Knopman and Petersen, 2014). However, a deeper analysis of the extant literature indicates a more complex mechanism. In general, understanding sex-specific trends in ADD and MCI-AD points to preclinical, demographic and pharmacological factors that could reveal onset and differential outcomes between males and females (Knopman and Petersen, 2014; Dubois et al., 2016). Pharmacological treatment of MCI-AD is limited (Petersen et al., 1999). Clinical trials involving a wide range of substances for MCI have failed to show efficacy on primary and secondary outcome parameters for treatments (Karakaya et al., 2013), suggesting that most treatments are targeted at AD. Several trials of cholinesterase inhibitors (ChEIs) have been conducted in individuals with amnestic type MCI, which is likely due to underlying AD (Tricco et al., 2013; Matsunaga et al., 2019). Cholinesterase inhibitors – donepezil, rivastigmine and galantamine are approved medications for the treatment of dementia due to AD (Dou et al., 2018), and outcomes have so far produced modest benefits (Li et al., 2019). Therefore, the decision to treat ADD patients with a ChEI is based on the likelihood that AD was the underlying etiology (Grossberg et al., 2019), indicating that other medications combined with a ChEI for the treatment of symptoms of other than those found in MCI-AD and ADD patients.

Preclinical data suggest that second-generation antipsychotics (SGAs) could reduce cognitive impairments (Goldberg et al., 2007; Hill et al., 2010). Whether there is evidence of sex differences in the use of SGAs as a pharmacological treatment option for MCI-AD and AD is not fully understood. Knowledge about the efficacy and limitations of the antidementive drugs and ChEIs in different stages of AD indicates a critical role for the serotonergic system in memory retention and learning by interacting with the cholinergic dopaminergic, γ-aminobutyric acid (GABA)ergic and glutaminergic systems (Seyedabadi et al., 2014). Selective serotonin reuptake inhibitors (SSRIs) are approved in the treatment of depressive disorders (Strawn et al., 2018), and fluoxetine an SSRI, is reported to enhance cognitive performance in AD (Xie et al., 2019). SSRIs selectively target the solute carrier family 6 member 4 responsible for terminating the action of serotonin in the synaptic cleft, consequently increasing neurotransmitter availability in the synapse (Mdawar et al., 2020). While SSRIs have emerged as promising therapies to delay the onset of cognitive deterioration in AD patients, it is not clear whether there are sex-specific differences in treating ADD and MCI-AD patients with SSRIs.

Since our sample was restricted to ADD and MCI-AD patients, we assumed that more females than males might

Abbreviations: AD, Alzheimer's disease; AChE, Acetylcholinesterase inhibitors; IRB, Institutional Review Board; APOE, Apolipoprotein E; CDR, clinical dementia rating; ChEI, Cholinesterase inhibitor; EOAD, Early-onset Alzheimer's disease; LOAD, Late-onset Alzheimer's disease; NSAIDs, non-steroidal anti-inflammatory drugs; NMDA, N-methyl-D-aspartate; ETOH, Ethanol; MMSE, mini mental exam; SSRIs, Selective Serotonin Reuptake Inhibitor; *AUROC,* Area Under the Receiver Operating Characteristics; ROC, receiver operating characteristic curve; OR, Odd ratio; SGA, second generation antipsychotics; VIF, Variance Inflation factor.

be affected, which is typical for the AD population (Viña and Lloret, 2010). Therefore, we hypothesized that males and females with ADD and MCI-AD differ regarding treatment with ChEIs or other medications including SSRIs and SGAs. The present study examined differences in patient demographics and pharmacological therapies in individuals treated with ChEIs, SSRIs and SGAs, and how this might contribute to sex differences between males and females with ADD and MCI-AD. Therefore, we determined sex-specific differences in ADD and MCI-AD patients undergoing ChEI, SSRIs and SGAs therapies. Moreover, since males and females present with differences in cognitive progression with females declining at much higher rates than males (Lin et al., 2015; Laws et al., 2016), we also determined specific demographic factors contributing to sex differences in patients who received ChEI, SSRIs, and SGAs.

TABLE 1 | Sex differences of demographic and clinical characteristics in mild cognitive impairment and dementia patients.

Characteristic	Male	Female	
Number of patients	**18153**	**27543**	***P*-value**
Age Group: No. (%)			
< 50	3283 (18.1)	4499 (16.3)	<0.001*[a]
50-59	1467 (8.1)	2687 (9.8)	
60-69	2777 (15.3)	3683 (13.4)	
70-79	4451 (24.5)	6066 (22.0)	
> = 80	6175 (34.0)	10608 (38.5)	
Mean ± SD	67.19 ± 22.00	69.72 ± 20.70	< 0.001*[b]
Race: No (%)			
White	14722 (81.1)	22300 (81.0)	< 0.001*[a]
Black	2465 (13.6)	4059 (14.7)	
Other	966 (5.3)	1184 (4.3)	
Hispanic Ethnicity: No. (%)	418 (2.3)	594 (2.2)	0.298
Tobacco	11402 (65.6)	11316 (41.9)	< 0.001*[a]
ETOH	6156 (35.6)	6784 (25.2)	< 0.001*[a]
Length of Stay	2.18 ± 7.63	1.70 ± 4.78	< 0.001*[b]
Medications			
Central acetylcholinesterase inhibitor	5425 (29.9)	7659 (27.8)	< 0.001*[a]
Donepezil	4943 (27.2)	6914 (25.1)	< 0.001*[a]
Galantamine	158 (0.9)	128 (0.5)	< 0.001*[a]
Rivastigmine	797 (4.4)	1197 (4.3)	0.820
Second Generation Antipsychotic	2447 (13.5)	4330 (15.7)	< 0.001*[a]
Aripiprazole	859 (4.7)	1582 (5.7)	< 0.001*[a]
Olanzapine	1091 (6.0)	1504 (5.5)	0.013*[a]
Risperidone	983 (5.4)	1894 (6.9)	< 0.001*[a]
Selective Serotonin Receptor Inhibitor	5140 (28.3)	9250 (33.6)	< 0.001*[a]
Citalopram	1835 (10.1)	3107 (11.3)	< 0.001*[a]
Escitalopram	3217 (17.7)	6019 (21.9)	< 0.001*[a]
Paroxetine	0 (0.0)	0 (0.0)	
Memantine	2880 (15.9)	4776 open (17.3)	< 0.001*[a]
Trazodone	0 (0.0)	0 (0.0)	
Buspirone	1272 (7.0)	3095 (11.2)	< 0.001*[a]
Valproate	0 (0.0)	0 (0.0)	

*Results for continuous variables are presented as Mean ± SD, while discrete data are presented as percentage frequency. Pearson's Chi-Square is used to compare sex differences between demographic and clinical characteristics in patients with mild cognitive impairment and dementia. [a]Pearson's Chi-Squared test; [b]Student's T test; *P-value < 0.05.*

MATERIALS AND METHODS

Study Population

Retrospective data of patients diagnosed with MCI-AD and ADD patients (early dementia associated AD) were retrieved from Alzheimer's database registry of the Prisma Health-Upstate (formerly known as Greenville Health System) from February 2016 to August 2021. The inclusion criteria were data from outpatients aged $\geq$ 40 years who met the requirements for the clinical diagnosis of MCI or early dementia, as defined in the DSMMD[26], and for possible AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (Cacabelos, 2007). Data for MCI-AD patients assessed using Mini-Mental State Exam (MMSE), with scores indicating mild cognitive impairment, were included in this study, while those with scores indicating more severe impairments were excluded. Data for patients not fulfilling the diagnostic criteria for AD and early dementia were also excluded. Pharmacological, social, and demographic risk factors were collected from a single database. Data for patients taking ChEIs including donepezil, galantamine, and rivastigmine, were also collected from this source. In addition, we obtained data for patients taking SSRIs including citalopram, escitalopram paroxetine, memantine, trazodone, buspirone, valproate, SGA medications including antipsychotics such as aripiprazole, olanzapine, and risperidone. Other variables included in this study were tobacco use and alcohol (ETOH) use. ETOH use was determined based off any past consumption of ETOH regardless of time and amount of consumption. Tobacco use was recorded in a similar fashion. Demographic factors included the age, race, and ethnicity of subjects.

Statistical Analysis

Univariate statistical analysis was used to determine demographic and pharmacological characteristics of patients with MCI-AD and ADD by sex. Discrete variables comparing MCI-AD and ADD patients were analyzed using the Man Whitney U or Pearson Chi-square test. Standard deviation, mean, and range were all calculated for continuous variables. The number and percentage of patients in that category were calculated for all discrete variables. The regression models were built using the established predictors from our univariate analysis using the backward selection method. This method was chosen because it allowed all the initially selected demographic and pharmacological risk factors to be included in the model and then systematically removed if they did not contribute to the overall significance of the model. The multicollinearity was determined for the interactive effects of variables using variance inflation factors (VIFs), with values > 5 has been reported to be suggestive of multicollinearity (Becker et al., 2015). Further, the validity of our model was tested using a Hosmer-Lemeshow test. The overall correct classification percentage and the area under the receiver

operating curve (AUROC) for score prediction were determined to test the model's sensitivity, specificity, and accuracy.

For each regression model, the dependent variables were MCI-AD or ADD, while the independent variables were the pharmacologic and demographic factors in patients with MCI-AD or ADD, stratified by sex. The regression models were developed separately for MCI-AD and ADD outcomes for males and females. Odd ratios at 0.5 significance level and 95% confidence interval (95% CI) were considered. The likelihood of being associated with MCI-AD or ADD was determined separately for male and female patients. The overall correct classification percentage and area under the Receiver Operating Curve (ROC) were used to determine the logistic regression model's sensitivity, specificity, and accuracy for male and female patients with MCI-AD or ADD. Statistical analyses were performed using SPSS software ver. 26.0 (IBM, Armonk, NY, United States).

RESULTS

A total of 45,696 AD Patients with 18,153 males and 27,543 females were included in this study. As shown in **Table 1**, females were more likely to present with increasing age (69.72 ± 20.70 vs. 67.19 ± 22.00), less likely to use tobacco (41.9 vs. 65.6%), and alcohol (25.2 vs. 35.6) than males. Additionally, females were less likely to be taking ChEIs (27.8 vs. 29.9%) including donepezil (25.1 vs. 27.2%) and galantamine (0.5 vs. 0.9%). However, females were more likely to be taking SGAs (15.7 vs. 13.5%), including aripiprazole (5.7 vs. 4.7%) and risperidone (6.9 vs. 5.4%), but less likely to take olanzapine (5.5 vs. 6.0%). They were also more likely to be treated with SSRIs (33.6 vs. 28.3%) than males, specifically, citalopram (11.3 vs. 10.1%) and escitalopram (21.9 vs. 17.7%), memantine (17.3 vs. 15.9), and buspirone (11.2 vs. 7.0%).

A total of 19,495 females presented with MCI-AD while 8,048 females presented ADD, whereas 13,569 males presented with MCI while 8048 males presented with ADD (**Table 2**). Females with ADD were more likely to be younger (84.35 ± 9.69 vs. 63.68 ± 21.02), Caucasian (82.1 vs. 80.5%), and less likely to be Hispanic (1.3 vs. 2.5%). Additionally, females with ADD presented with lower rates of tobacco (37.2 vs. 43.9%) and ETOH (12.8 vs. 38.4%) use. Higher lengths of stay (2.16 ± 4.22 vs. 1.51 ± 4.98) were found in females with dementia compared to MCI. They were also more likely to take ChEIs (51.4 vs. 18.1%), specifically donepezil (45.6 vs. 18.7%), galantamine (0.8 vs. 0.3%), and rivastigmine (9.3 vs. 2.3%). In addition, females with ADD were more likely to take SGAs (20.5 vs. 13.8%) including aripiprazole (4.8 vs. 6.1%), olanzapine (7.9 vs. 4.5%), risperidone (10.8 vs. 5.3%), and SSRIs (35.4 vs. 32.8%) such as citalopram (12.6 vs. 10.8%), escitalopram (23.9 vs. 21.0%), and memantine (34.8 vs. 10.1%) with the exception of buspirone (8.2 vs. 12.5%).

Males with ADD were more likely to be older (81.08 ± 10.86% vs. 62.49 ± 22.82%) and Caucasian (81.3 vs. 81.0%), and less likely to be Hispanic (1.1 vs. 2.7%), with higher rates of tobacco use (69.5 vs. 64.2%) and lower rates of ETOH use (25.7 vs. 39.1%). Males with ADD were also more likely to take ChEIs (52.2 vs. 22.3%), specifically donepezil (46.6 vs. 20.7%), galantamine (1.3 vs. 0.7%), and rivastigmine (8.9 VS. 2.9%). They were more likely to take SGAs (18.5 vs.11.8%) including olanzapine (9.1 vs. 5.0%), risperidone (8.9 vs. 4.3%), and SSRIs (30.3 vs. 27.7%) including citalopram (11.6 vs. 9.6%), escitalopram (19.3 vs. 17.2%), memantine (33.0 vs. 10.1% and buspirone (6.0 vs. 7.3%) with the exception of (aripiprazole (3.5 vs. 5.1%).

Figure 1 shows demographic and pharmacological factors associated with MCI-AD and ADD in male patients. Galantamine [OR = 0.559, 95% CI, 0.382 – 0.818, P = 0.003], and Hispanic ethnicity [OR = 0.166, 95% CI, 0.020 – 1.355, P = 0.094] were associated with MCI. Length of stay [OR = 1.009, 95% CI, 1.003 – 1.015, P = 0.005], increasing age [OR = 1.075, 95% CI, 1.071 – 1.079, $P < 0.001$], donepezil [OR = 1.639, 95% CI, 1.503 – 1.787, $P < 0.001$], rivastigmine [OR = 1.394, 95% CI, 1.184 – 1.642, $P < 0.001$], memantine [OR = 2.235, 95% CI, 2.021 – 2.473, $P < 0.001$], aripiprazole [OR = 1.360, 95% CI, 1.080 – 1.712, P = 0.009], olanzapine [OR = 2.727, 95% CI, 2.315 – 3.212, $P < 0.001$], risperidone [OR = 2.973, 95% CI, 2.506 – 3.526, $P < 0.001$], citalopram [OR = 1.187, 95% CI, 1.044 – 1.350, P = 0.009] African American racial group [OR = 2.380, 95% CI, 2.120 – 2.674, $P < 0.001$], and a history of tobacco use [OR = 1.150, 95% CI, 1.054 – 1.254, P = 0.002] were associated with dementia. The predictive power of the regression model was moderately strong. The area under the curve (AUORC) is 0.805, 95% CI, $P < 0.001$.

The factors associated with MCI-AD and ADD in females were also determined (**Figure 2**). Buspirone [OR = 0.767, 95% CI, 0.683 – 0.861, $P < 0.001$] and a history of ETOH use [OR = 0.484, 95% CI, 0.442 – 0.529, $P < 0.001$] were associated with MCI. Length of stay [OR = 1.028, 95% CI, 1.020 – 1.035, $P < 0.001$], increasing age [OR = 1.096, 95% CI, 1.093 – 1.100, $P < 0.001$], donepezil [OR = 2.185, 95% CI, 2.035 – 2.346, $P < 0.001$], galantamine [OR = 1.589, 95% CI, 1.051 – 2.403, P = 0.028] rivastigmine [OR = 1.372, 95% CI, 1.192 – 1.579, $P < 0.001$] memantine [OR = 2.283, 95% CI, 2.104 – 2.477, $P < 0.001$] aripiprazole [OR = 1.807, 95% CI, 1.544 – 2.113, $P < 0.001$] olanzapine [OR = 2.289, 95% CI, 1.986 – 2.640, $P < 0.001$] risperidone [OR = 2.548, 95% CI, 2.246 – 2.889, $P < 0.001$], escitalopram [OR = 1.213, 95% CI, 1.119 – 1.315, $P < 0.001$] African American racial group [OR = 1.395,1.268 – 1.535, $P < 0.001$] and a history of tobacco use [OR = 1.150, 95% CI, 1.073 – 1.233, $P < 0.001$] were associated with ADD. The strength of the model was found to be moderately strong. The area under the curve (AUORC) is 0.853, 95% CI, P < 0.001.

DISCUSSION

ADD and MCI-AD patients represent a significant clinical group as they are at increased risk of worsening cognitive functions and are an ideal target for therapeutic interventions. Since biological changes typical of AD have been found in MCI patients (Frisoni et al., 2010), and pharmacological treatment of MCI due to AD is limited, the decision to treat patients with a ChEI would depend on whether an underlying etiology of AD could be assessed.

TABLE 2 | Demographic and clinical characteristics of mild cognitive impairment versus dementia in patients stratified by sex.

	Male			Female		
Characteristic	**Mild Cognitive Impairment**	**Dementia**		**Mild Cognitive Impairment**	**Dementia**	
Number of patients	**13569**	**4584**	***P*-value**	**19495**	**8048**	***P*-Value**
Age Group: No. (%)						
< 50	3230 (23.8)	53 (1.2)	<0.001*[a]	4463 (22.9)	36 (0.4)	<0.001*[a]
50-59	1375 (10.1)	92 (2.0)		2644 (13.6)	43 (0.5)	
60-69	2351 (17.3)	426 (9.3)		3227 (16.6)	456 (5.7)	
70-79	3301 (24.3)	1150 (25.1)		4421 (22.7)	1645 (20.4)	
> = 80	3312 (24.4)	2863 (62.5)		4740 (24.3)	5868 (72.9)	
Mean ± SD	62.49 ± 22.82	81.08 ± 10.86	< 0.001*[b]	63.68 ± 21.02	84.35 ± 9.69	< 0.001*[b]
Race: No (%)						
White	10993 (81.0)	3729 (81.3)	< 0.001*[a]	15696 (80.5)	6604 (82.1)	< 0.001*[a]
Black	1743 (12.8)	722 (15.8)		2887 (14.8)	1172 (14.6)	
Other	833 (6.1)	133 (2.9)		912 (4.7)	272 (3.4)	
Hispanic Ethnicity: No. (%)	366 (2.7)	52 (1.1)	< 0.001*[a]	491(2.5)	103 (1.3)	< 0.001*[a]
Tobacco	8281 (64.2)	3121 (69.5)	< 0.001*[a]	8389 (43.9)	2927 (37.2)	< 0.001*[a]
ETOH	5003 (39.1)	1153 (25.7)	< 0.001*[a]	5775 (30.4)	1009 (12.8)	< 0.001*[a]
Length of Stay	2.09 ± 7.79	2.45 ± 7.13	0.006*[b]	1.51 ± 4.98	2.16 ± 4.22	< 0.001*[b]
Medications						
Central acetylcholinesterase inhibitor	3032 (22.3)	2393 (52.2)	< 0.001*[a]	3522 (18.1)	4137 (51.4)	< 0.001*[a]
Donepezil	2807 (20.7)	2136 (46.6)	< 0.001*[a]	3246 (16.7)	3668 (45.6)	< 0.001*[a]
Galantamine	100 (0.7)	58 (1.3)	< 0.001*[a]	67 (0.3)	61 (0.8)	< 0.001*[a]
Rivastigmine	388 (2.9)	409 (8.9)	< 0.001*[a]	447(2.3)	750 (9.3)	< 0.001*[a]
Second Generation Antipsychotic	1598 (11.8)	849 (18.5)	< 0.001*[a]	2682 (13.8)	1648 (20.5)	< 0.001*[a]
Aripiprazole	698 (5.1)	161 (3.5)	< 0.001*[a]	1198 (6.1)	384 (4.8)	< 0.001*[a]
Olanzapine	672 (5.0)	419 (9.1)	< 0.001*[a]	871(4.5)	633 (7.9)	< 0.001*[a]
Risperidone	577 (4.3)	406 (8.9)	< 0.001*[a]	1026 (5.3)	868 (10.8)	< 0.001*[a]
Selective Serotonin Receptor Inhibitor	3753 (27.7)	1387 (30.3)	< 0.001*[a]	6397 (32.8)	2853 (35.4)	< 0.001*[a]
Citalopram	1301 (9.6)	534 (11.6)	< 0.001*[a]	2096 (10.8)	1011 (12.6)	< 0.001*[a]
Escitalopram	2333 (17.2)	884 (19.3)	0.001*[a]	4097 (21.0)	1922 (23.9)	< 0.001*[a]
Paroxetine	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Memantine	1365 (10.1)	1515 (33.0)	< 0.001*[a]	1973 (10.1)	2803 (34.8)	< 0.001*[a]
Trazodone	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Buspirone	996 (7.3)	276 (6.0)	0.002*[a]	2436 (12.5)	650 (8.2)	< 0.001*[a]
Valproate	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	

*Results for continuous variables are presented as Mean ± SD, while discrete data are presented as percentage frequency. Pearson's Chi-Square is used to compare differences between demographic and clinical characteristics in groups either mild cognitive Impairment or dementia stratified by sex. [a]Pearson's Chi-Squared test; [b]Student's T test; *P-value < 0.05.*

The current study evaluated sex differences in ADD and MCI-AD patients treated with ChEI, SSA, and SGA therapies. In the univariate analysis, our findings revealed that more female patients presented with ADD and MCI-AD when compared with males. Moreover, MCI-AD females were more likely to be taking SGAs, including aripiprazole and risperidone but less likely to take olanzapine. In addition, females were more likely to be treated with SSRIs, specifically citalopram escitalopram, memantine, and buspirone.

In the adjusted analysis for males, Hispanic males taking ETOH treated with galantamine were associated with MCI-AD. In contrast, African American males with an increasing length of stay for treatment, increasing age treated with donepezil, rivastigmine, memantine, aripiprazole, olanzapine, risperidone and citalopram were associated with ADD. For females, buspirone and a history of ETOH use were associated with MCI-AD, while African American females, and an increased length of stay, increasing age, and treatment with donepezil, galantamine, rivastigmine, memantine, aripiprazole, risperidone, buspirone and escitalopram were associated with ADD.

Pharmacologic treatments for AD with donepezil, galantamine, and rivastigmine include targeting the primary manifestations that have cognitive impairments observed in both ADD and MCI-ADD patients (Cacabelos, 2007). In general, ChEIs reduce acetylcholine breakdown in the brain and are considered a treatment option for AD. They also offer a feasible therapeutic target to stabilize cognitive functions (Stanciu et al., 2020). Donepezil, is a ChEI known to improve

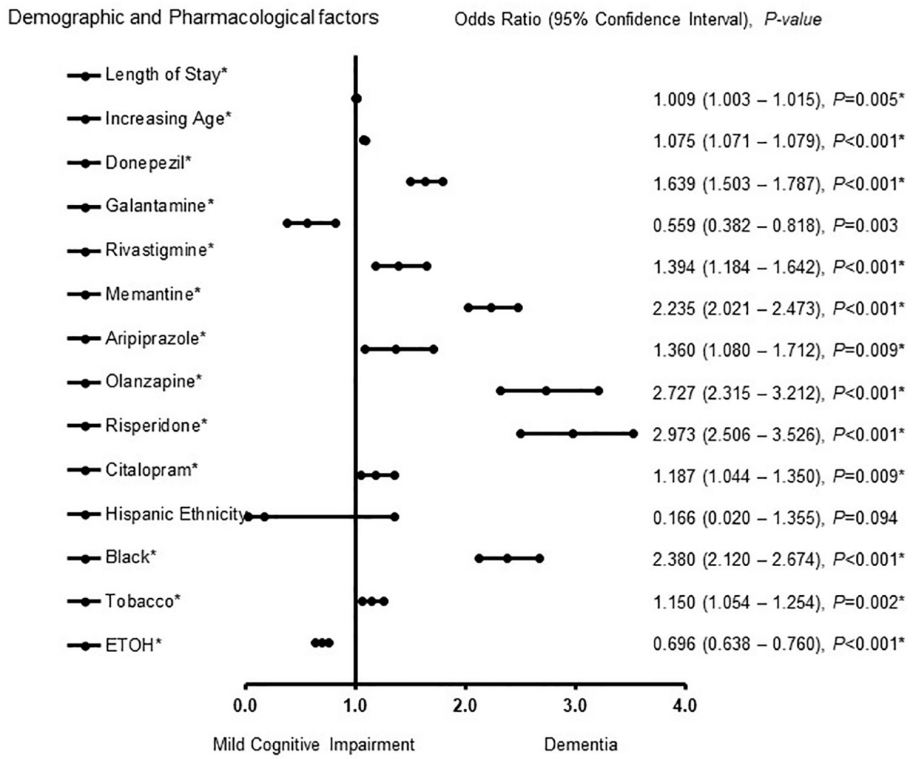

FIGURE 1 | Demographic and pharmacological factors associated with mild cognitive impairment and Alzheimer patients with dementia in males. Adjusted OR < 1 denote factors that are associated with mild cognitive impairment while OR > 1 denote factors that are associated with dementia. Hosmer-Lemeshow test ($P < 0.001$*), Cox & Snell ($R^2 = 0.217$). The overall classified percentage of 76.6% was applied to check for fitness of the logistic regression model. *Indicates statistical significance ($P < 0.05$) with a 95% confidence interval.

cerebral blood flow (CBF) to enhance memory (Kogure et al., 2017). Rivastigmine is a brain-selective inhibitor of "pseudo-irreversible" AChE, and its metabolism is independent of the cytochrome P450 system (Li et al., 2019). Galantamine is a newly available cholinergic drug that counteracts AD by specifically and reversibly inhibiting acetylcholinesterase (AChE) and altering the nicotinic cholinergic receptors, thereby reducing central cholinergic neurotransmission (Li et al., 2019).

Treatment outcomes of ChEIs are reported to be controversial. For example, donepezil, galantamine, and rivastigmine are reported to stabilize or slow the decline in cognition and improve cognition for donepezil- compared with galantamine-treated patients (Jones et al., 2004). Adjusted indirect comparisons suggest that donepezil and rivastigmine may be slightly more efficacious than galantamine (Hansen et al., 2008). Other studies indicate that galantamine has potent therapeutic effects on all aspects of AD, but donepezil and rivastigmine do not have effective therapeutic effects on some aspects of cognitive function (Li et al., 2019). We observed that males with MCI-AD were only treated with galantamine. In contrast, females with MCI-AD did not receive any ChEIs, indicating differences in the use of ChEIs as a treatment option for males and females with MCI-AD.

While our current data cannot explain the reason that females with MCI-AD were not treated with any ChEIs therapies in our data set, ChEIs are reported to slightly delay the loss of brain function in people who have mild to moderate AD; however, they do have side effects such as nausea, dizziness, vomiting, diarrhea, dizziness, asthenia and anorexia, all symptoms linked to cholinergic overstimulation (Imbimbo, 2001). Therefore, the adverse events may outweigh the benefits, such that ChEIs produce a small gift on several cognitive function scales (Hitzeman, 2006; Bailey-Taylor et al., 2022) in female patients. Moreover, our finding that males and females with ADD were more likely to receive donepezil, galantamine, and rivastigmine reveals a robust comparable approach in using ChEIs for ADD males and females. Since females present with higher rates of clinically diagnosed cases of dementia and AD (Beam and Kim, 2020), more excellent longitudinal rates of cognitive and functional decline in MCI than males (Tarawneh and Holtzman, 2012), a comparable robust approach in ChEIs may offer a robust approach to manage and reduce the high rates of ADD in females.

MCI-AD and ADD are not a part of the normal aging process (Lo, 2017). In individuals with ADD, an impairment in cognitive function that results in mental decline that is sufficiently severe to disrupt their activities of daily life (Popp et al., 2015). The cognitive efficacy of antipsychotics has gained more research attention in recent years, as aripiprazole (Kohen et al., 2010), risperidone, or olanzapine (Vigen et al., 2011)

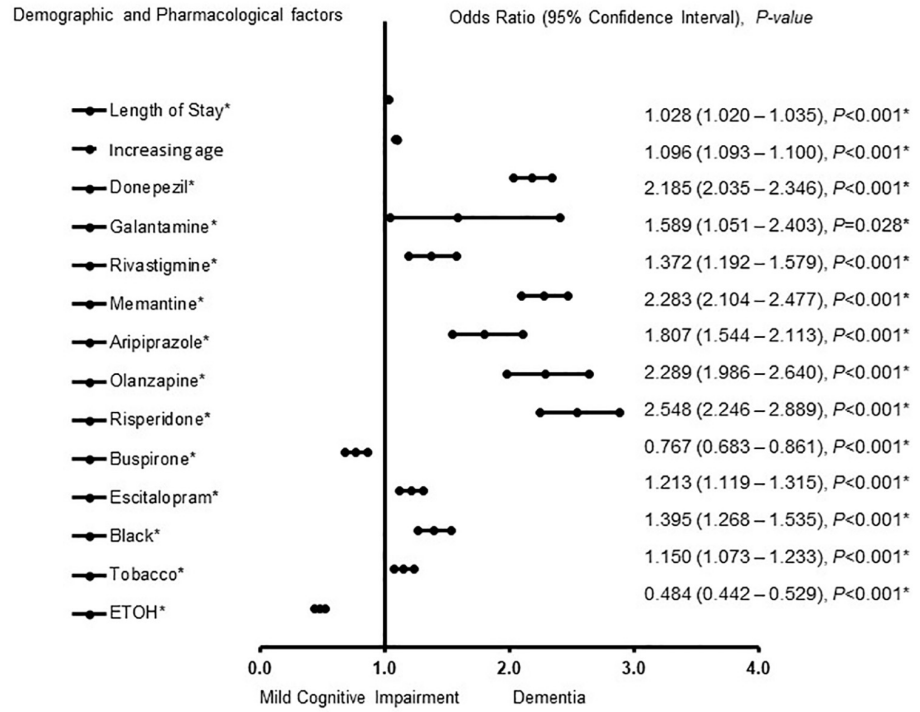

FIGURE 2 | Demographic and pharmacological factors that were associated with mild cognitive impairment and Alzheimer patients with dementia in females. Adjusted OR < 1 denote factors that are associated with mild cognitive impairment while OR > 1 denote factors that are associated with dementia. Hosmer-Lemeshow test ($P < 0.001$*), Cox & Snell ($R^2 = 0.307$). The overall classified percentage of 70.7% was applied to check for fitness of the logistic regression model. *Indicates statistical significance ($P < 0.05$) with a 95% confidence interval.

improved cognitive functions in ADD patients. Loss of cognitive functions in dementia patients is characterized by a loss of more than one cognitive domain including learning and memory, language, visuo-spatial, executive and psychomotor (Tarawneh and Holtzman, 2012). In contrast, for MCI, only one of these domains must be impaired to make a diagnosis (Tarawneh and Holtzman, 2012). Our finding that SGAs including aripiprazole, olanzapine, risperidone were administered to both males and females with ADD is supported by previous studies (Seeman, 2004; Fulone et al., 2021), which found that antipsychotic use should not differentiate between male and female ADD patients. However, human studies (Beierle et al., 1999; Pérez et al., 2003) have shown that the pharmacokinetics and the pharmacodynamics of drugs differ in females and males and are influenced by sex-specific factors such as body habitus, diet, concurrent medications, and hormonal transitions. Furthermore, neurotransmitter levels diminish with age at different rates in females than in males (Peters, 2006). Some antipsychotic drug treatments have side effects, such as weight gain, which is more worrisome for females than males (Seeman, 2020). Therefore, while females may require same antipsychotic medication like males to achieve a better outcome, it may be at the expense of a higher side effect burden, precisely hormonal and metabolic side effects (Seeman, 2004). Therefore, sex-specific treatment regimens need to be developed to optimize outcomes in the use of SGAs for ADD patients.

We observed that males with ADD were more likely to be treated with memantine and citalopram, while females were more likely to be treated with memantine, buspirone, and escitalopram. While cholinergic dysfunction was long thought to be the sole contributor to AD symptomatology (Mdawar et al., 2020), growing evidence supports the contributory role of a dysfunctional monoaminergic system (Šimić et al., 2017). The serotonergic system plays a pivotal role in memory retention and learning by interacting with the cholinergic, dopaminergic, γ-aminobutyric acid (GABA)ergic and glutaminergic systems (Kandimalla and Reddy, 2017). Buspirone, escitalopram and citalopram are serotonin norepinephrine reuptake inhibitors (SNRIs) commonly used in elderly males and females, due to their tolerability and safety profile (Crocco et al., 2017). Memantine, a low-affinity non-competitive NMDA receptor antagonist, is the only glutamatergic drug approved for the treatment of moderate to severe cognitive symptoms of AD (Tariot et al., 2004). Memantine can be used in addition to acetylcholinesterase inhibitors in patients with AD (Scarpini et al., 2003). This specific combination is reported to delay the progression of dementia by preventing the pathological activation of NMDA receptors (Parsons et al., 2013). Therefore, our finding supports existing studies (Scarpini et al., 2003; Parsons et al., 2013) that memantine can be used for the initial therapy of cognitive functions in dementia patients.

Several studies support our finding that African American males and females with a history of ETOH use, increasing length of stay for treatment, and increasing age were associated with dementia (Langa et al., 2017). The higher rates of dementia among African-Americans contribute to an increased length of stay for care (Hill et al., 2015). There is also evidence that females have higher rates of dementia than males, mainly because females live longer (Mensah et al., 2005). Therefore, while disparities may be reduced by increasing levels of cognitive reserve and management of disease among blacks (Chen and Zissimopoulos, 2018), there also remains a complex combination of socioeconomic and cultural factors associated with these disparities. Health disparities often are seen through the lens of access to care or resources. However, a lack of diversity in clinical therapeutic development means that surmounting access barriers will not reduce disparities if therapeutics target only a small fraction of the diverse population. Future studies on factors associated with racial/ethnic differences in dementia risk should also focus on treatment options for racial and ethnic minorities by recruiting various participants into clinical trials of existing or new therapeutics.

LIMITATIONS

Since this is a retrospective study, some potential limitations should be considered while interpreting the results of this study. The retrospective data were from a single institution; therefore, the results cannot be extrapolated to other institutions. In addition, data collection using electronic medical records could introduce human error as data from some study patients may have been excluded, which could have altered the results. Moreover, data on the systematic evaluation of behavioral disorders was not available since behavioral disorders, and the drugs used to treat them are usually considered for the prescription of ChEIs and other medications. Also, data on MMSE and CDR were not available to determine disease progression and behavioral alterations. In addition, information on the duration for the ChEIs, SSRSs, SGAs and data for apolipoprotein E (APOE) was not included in the database. Analyzing apolipoprotein E (APOE) in future studies will help determine sex differences or similarities and an increased risk at younger ages. It will also help to determine whether E (APOE) may contribute to cognitive change and differ across different demographic groups. All of our subgroup analyses were predetermined, and our analyses were repeated several times to eliminate the possibility of type 1 statistical errors. Finally, while this is a single study, the demonstration of consistent sex disparities in the demographic and pharmacological characteristics increases the generalizability of our findings.

CONCLUSION

In our findings, Hispanic males treated with galantamine were associated with MCI, while African American males with the increasing length of stay, increasing age, and treated with donepezil, rivastigmine, memantine, aripiprazole, olanzapine, risperidone and citalopram were associated with dementia. For females, Buspirone and history of ETOH use were associated with MCI. In contrast, African American females, with an increased length of stay, increasing age, treatment with donepezil, galantamine, rivastigmine, memantine, aripiprazole, risperidone, buspirone, and escitalopram were associated with MCI-AD. Therefore, we observed differences and similarities in demographic factors and pharmacological therapies for males and females with MCI and dementia AD patients. These findings will hopefully facilitate developments in pharmacological treatment options of cognitive symptoms of dementia and MCI impairment due to AD in future studies. Furthermore, our results highlight the importance of taking sex into account in the clinical trials for ChEI, SGAs and SSRIs pharmacological agents for MCI and dementia patients with AD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This is a retrospective data collection. This study was approved by the Institutional Review Board of PRISMA Health institutional committee for ethics (approval number: 00052571). All data were fully anonymized before they were accessed. Patients' data used in our retrospective analysis were from PRISMA Health Alzheimer data registry. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

OC-A, SN, MB-T, NP, LR, and TN designed the concept, experiment and data analysis. At the same time, RG, BM and RR-S critically revised the drafts, interpreted the results, read and approved the last version of this manuscript. All authors have read and approved the manuscript.

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Sex Differences in Spatial Learning and Memory in Valproic Acid Rat Model of Autism: Possible Beneficial Role of Exercise Interventions

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In the current study, we first tried to determine sex differences in spatial learning and memory in the valproic acid (VPA) rat model of autism. Second, the effects of interval training (IT) and continuous training (CT) exercises were examined in male and female offsprings. To induce autism-like animal model, the pregnant rats were injected 500 mg/kg NaVPA (intraperitoneal) at the embryonic day 12.5. IT and CT aerobic exercises were started at postnatal day 56. Then, on postnatal days 84–89, a Morris water maze (MWM) test was conducted on the separate groups of offsprings. Aerobic training was performed on a rodent treadmill with 0% slope for 8 weeks, 5 days/week, and 50 min/day. Unlike control animals, VPA-exposed female offspring had a better performance than VPA-exposed male offspring in MWM acquisition. In the case of MWM reference memory, we did not observe a sex difference between VPA-exposed male and VPA-exposed female offspring. Both IT and CT exercises in both control and VPA-exposed male rats significantly improved MWM acquisition. Moreover, both IT and CT exercises significantly improved MWM acquisition in control female rats. In addition, IT exercise (but not CT) significantly improved MWM acquisition in VPA-exposed female offsprings. Both IT and CT exercises in VPA-exposed that male and female offsprings improved the MWM reference memory. In conclusion, our observation demonstrated that prenatal exposure to VPA affects the spatial learning and memory in a sex dependent manner. We have shown that both IT and CT exercises are able to improve cognitive function in healthy and autistic rat offsprings.

Keywords: valproic acid, spatial memory, sex difference, exercise, autistic rats

INTRODUCTION

Autism spectrum disorder (ASD) is a prototypic neurodevelopmental disorder that has the following characteristics: deficit in social interaction, language impairment and communication disorder, stereotyped and repetitive behaviors, and sometimes restricted interests and activities (Kientz and Dunn, 1997; American Psychiatric Association, 2013; Ousley and Cermak, 2014).

Research has shown that taking antiepileptic drugs, such as valproic acid (VPA) in pregnant women is associated with an increased risk of developing ASD in their children (Rasalam et al., 2005; Bromley et al., 2013; Silvestrin et al., 2013). Studies in rodents have shown that maternal exposure to VPA rises the risk of offspring with autism, producing an animal model of ASD that reflects the various characteristics of patients with ASD (Campolongo et al., 2018; Nicolini and Fahnestock, 2018; Bossu and Roux, 2019). In general, an intraperitoneal administration of VPA into pregnant female rats induces autistic symptoms in the offsprings, and the structures of the brain and the levels of biomarkers in the brain and blood of these offsprings are similar to those of patients with autistic (Christensen et al., 2013; Ornoy et al., 2015).

Finding sex differences in different brain functions looks necessary because the male and female nervous systems respond differently to abnormal physiological conditions (Sickmann et al., 2014). On the other hand, studies have shown that gender may have a significant consequence on cognitive functions in humans (Cahill, 2006; Beggiato et al., 2017) and the behavioral performances of rat (Schneider et al., 2008). Different strategies for decision-making and memory encoding have been reported in men and women.

Men are 4–7 times more likely than women to develop autism (Fombonne, 2009). The male bias in ASD has led to women with ASD being under-researched. Although gender disparity has been reported in ASD, there is little research on gender related to the spatial learning and memory in ASD. It has been shown that the phenotype of women with ASD may be different from that of men (Rivet and Matson, 2011). According to the literature, there are many contradictions regarding the effect of VPA on learning and memory, from "spatial learning and memory enhancement" (Edalatmanesh et al., 2013) to "spatial learning and memory impairments" (Gao et al., 2016; Wu et al., 2018). In the current work, we first tried to determine sex differences in spatial learning and memory in a VPA rat model of autism in the Morris water maze (MWM) test. Second, the effects of interval and continuous exercise training were examined in male and female offspring.

Research has shown that exercise can be used as an effective non-pharmacological strategy to reduce the complications of autism. Exercise increases the cognitive ability, prevents aging-induced failure of memory, protects against neuronal damage, and reduces the symptoms of neurodevelopmental and neuropsychiatric disorders (Cotman et al., 2007; Kim et al., 2010, 2011). Some works have revealed that exercise interventions may be a vital mediator in the treatment of neuropsychiatric, such as autism (Petrus et al., 2008; Lang et al., 2010; Kim et al., 2011).

Treadmill exercise has been shown to improve behavioral outcomes in autistic rats by upregulating the reelin signaling pathway (Seo et al., 2013). However, it is not yet clear what type and intensity of exercise training can have the best effects on ASD. Despite the physiological and clinical relevance of exercise training on autism, no studies have so far provided evidence on the comparisons between the impacts of training regimes of disparate intensities but the same volume on cognition ability. Therefore, in the present study, the effect of interval training (IT) and continuous training (CT) aerobic exercises were examined in male and female VPA-exposed offspring.

MATERIALS AND METHODS

Experimental Animals and the Valproic Acid Rat Model of Autism

Ethical approval was received by the Animal Study Ethics Committee of our university (Ethics Code: IR.UMSHA.REC.1397.931). In addition, all experiments were done in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Every effort was made to minimize suffering. Two female Wistar rats mated overnight with an adult male (i.e., 6 weeks of age) of the same strain for pregnancy. The number of impregnated dams was 15. Coition was confirmed at the following morning [on embryonic day 0 (E0)] by the presence of a vaginal plug or sperm in the vaginal smear. Sodium valproate (NaVPA, Sigma) was dissolved in saline to a concentration of 150 mg/ml (to induce a rat model of autism). On E12.5, VPA-dams (n = 8) received single intraperitoneal (i.p.) injection of NaVPA (500 mg/kg, 3.3 ml/kg) (Hajisoltani et al., 2019); saline-dams (n = 7) received a single injection of saline as vehicle (i.p., 3.3 ml/kg). The rats room temperature was 22 ± 2°C with a light-dark cycle (12 h light–12 h dark), and the animals had free access to tap water and standard laboratory chow. The dams were housed separately and allowed to grow their own litters. On postnatal day 21, the male and female offspring rats were randomly assigned to six groups (n = 6–10 in each group): the control group, control plus IT exercise (Control-IT), control plus CT exercise (Control-CT), VPA-exposed animals (VPA), VPA-exposed animals plus IT exercise (VPA-IT), and VPA-exposed animals plus CT exercise (VPA-CT). The total number of animals in all groups was 94 male rats and female rats. **Figure 1** shows the experimental design and timetable of the work.

Training Program

Aerobic training in both interval and continuous exercise groups was performed on a rodent treadmill with 0% slope for 8 weeks, 5 days/week, and 50 min/day (Pereira et al., 2013; Nunes et al., 2015). Before starting the training program from the 8 week old-age of the rats, the animals participated in a treadmill acclimatization period for 5 days, 10–30 min/day at a speed of 10–15 m/min (Nunes et al., 2015). Then, a graded exercise test was performed on the treadmill to achieve maximal running speed in the rats. The initial speed in the test was 10 m/min which was increased by 1 m/min every minute until the rats reached their final speed and could no longer continue the activity; so, the obtained final speed on the treadmill was considered as maximal running speed (Pereira et al., 2013). During the main training sessions, the treadmill speed in the continuous group was a constant load of 70% maximum throughout the training time. The animals in the interval group trained at 1 min high-intensity intervals with 90% maximum speed alternating by 1 min medium-intensity intervals with 50% maximum speed until completing 50 min training time/day (Pereira et al., 2013;

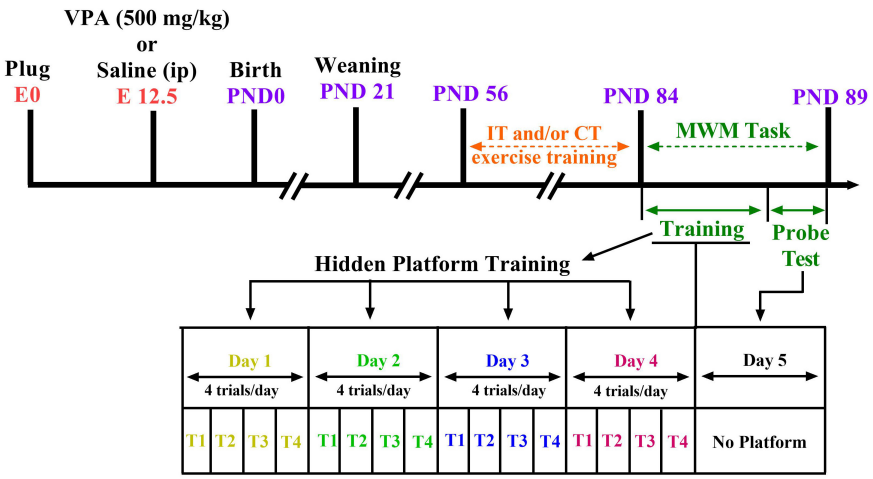

FIGURE 1 | Study design and time diagram of Morris water maze (MWM) task. Two female rats with one male rat were caged overnight and allowed to mate. The first day of pregnancy was determined by the presence of sperm in vaginal smear. On E12.5, valproic acid (VPA)-dams (n = 8) received single intraperitoneal (i.p.) injection of NaVPA (500 mg/kg, 3.3 ml/kg); saline-dams (n = 7) received a single injection of saline as vehicle (i.p., 3.3 ml/kg). Offsprings were weaned on P21. Interval exercise training (IT) and continuous exercise training (CT) were started at postnatal day 56. Then, on postnatal days 84–89, the MWM test was conducted on the separate groups of pups.

Nunes et al., 2015). Thus, the training volume was equal in both groups so that the final results of the research could be compared between them.

Morris Water Maze Task

Apparatus

The Morris water maze (MWM) navigation test, as a hippocampal-dependent test, is widely used to assess spatial learning and memory (Karimi et al., 2017; Habibitabar et al., 2020). Distinguishing between spatial conditions (hidden platform) and non-spatial conditions (visible platform) is the main advantage of the MWM task. In addition, the MWM test environment reduces odor trail interference. The MWM consists of a circular black tank (diameter = 155 cm, height = 60 cm) divided into four equal quadrants and filled with water (35 cm deep and 22 ± 1°C). An invisible platform made of transparent plexiglass (diameter = 10 cm) was placed 2 cm below the water surface in the center of the eastern quadrants (as a target quadrants). A video camera, computer, and tracking software (CCD camera, Panasonic Inc., Japan) were used to record the swim path and performance of rats for further analysis. Large posters prints were used on the wall of the room as visual cues.

Habituation

Furthermore, 1 day before the training sessions, the rats swam for 1 min in a tank without a platform to adapt to the MWM test.

Hidden Platform Training

The training sessions were conducted according to our previous studies (Karimi et al., 2017; Habibitabar et al., 2020). Briefly, in the training sessions one block of four trials per day was conducted for 4 consecutive days. Each trial started by placing the rat in the middle of one of the four quadrants. Each rat had 90 s swimming time to find the hidden platform. If the rat did not find the hidden platform during this time, it was manually moved to the platform by the experimenter. The time between the two consecutive trials was 10 min. Time to reach the platform (escape latency) and swimming distance during training days were recorded to assess the acquisition of the MWM task. The daily average of all trials from day 1 to 4 was used in our analysis.

Spatial Reference Memory (Probe Test or Retention)

The spatial retention test was conducted 24 h after the last training trial and rats swam for 60 s in a tank without platform. The rats were put in the western quadrant (i.e., exactly opposite from where the platform was placed in the training days) and the time spent and distance traveled in target zone was recorded.

Visual Test

A visual test was conducted 30 min after the retention test. For this test, the platform was set 1 cm above the water level (in a different zone) and covered with bright color aluminum so that the rat could locate the platform (for 60 s) using a local visual stimulus rather than spatial cues. All tests were conducted between 12:00 and 14:00.

Statistical Analysis

GraphPad Prism® 8.0.2 software was used for statistical analysis. Shapiro–Wilk test was used to check the data normality. All data passed normality test (Shapiro–Wilk test was greater than 0.05), so we used one-way and two-way repeated-measures ANOVA analysis followed by a *post hoc* analysis (Newman–Keuls and the Bonferroni multiple comparison tests, respectively). Next, for quantificational evaluation, the area under curve (AUC) was calculated for escape latency and traveled distance, which represented the general cognitive level over 4 consecutive days. The data were presented as mean ± SEM. Differences were considered statistically significant at $p < 0.05$.

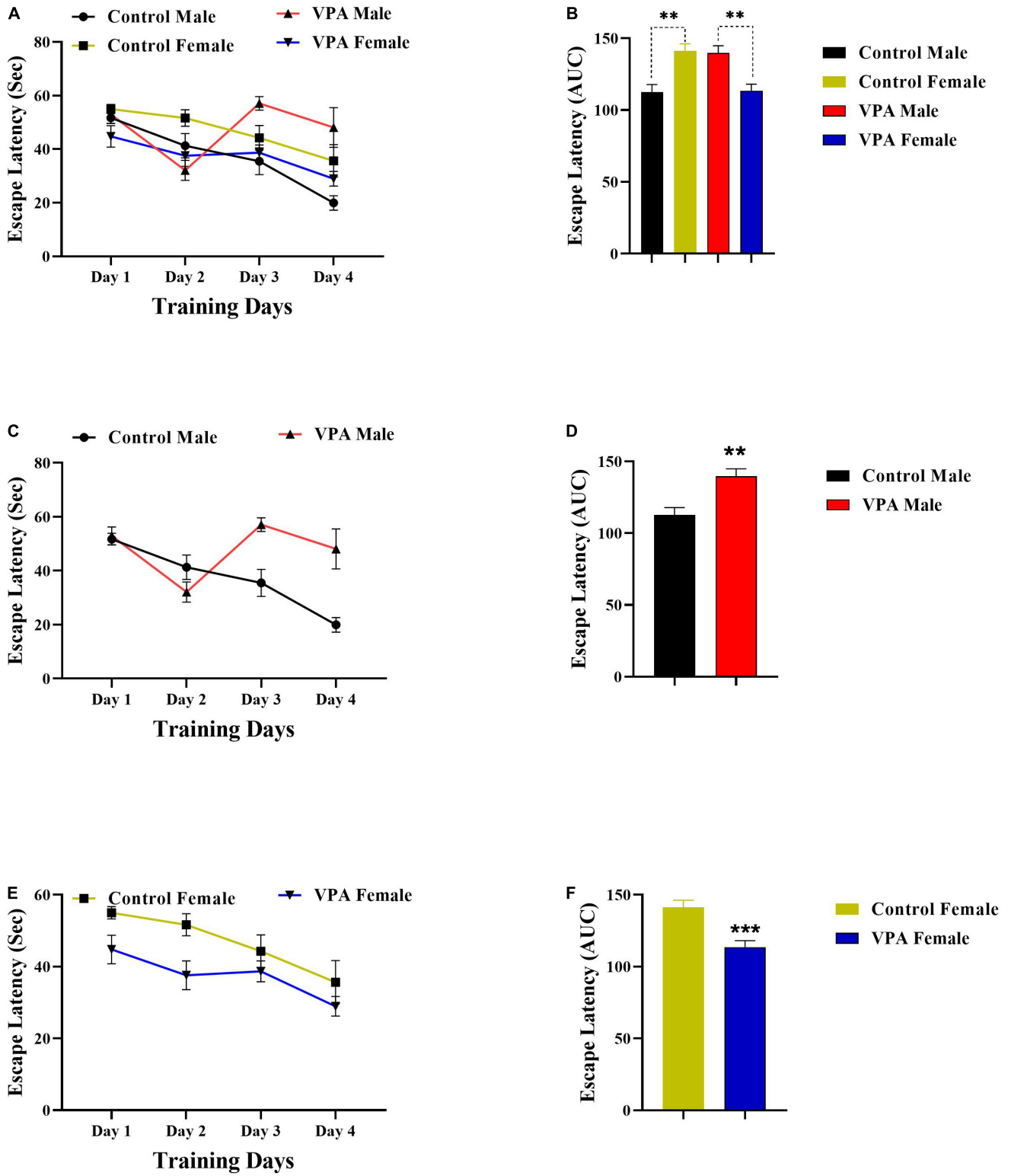

FIGURE 2 | Latency to find a hidden platform during training days. **(A)** Significant sex differences in the escape latency in control and VPA-exposed male and female offspring. **(B)** Area under the curve (AUC) for escape latency. **(C)** Significant differences in the escape latency in control and VPA-exposed male offspring. **(D)** AUC for escape latency in male offsprings. **(E,F)** Same analysis as **(C,D)**, respectively, but for female offsprings. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

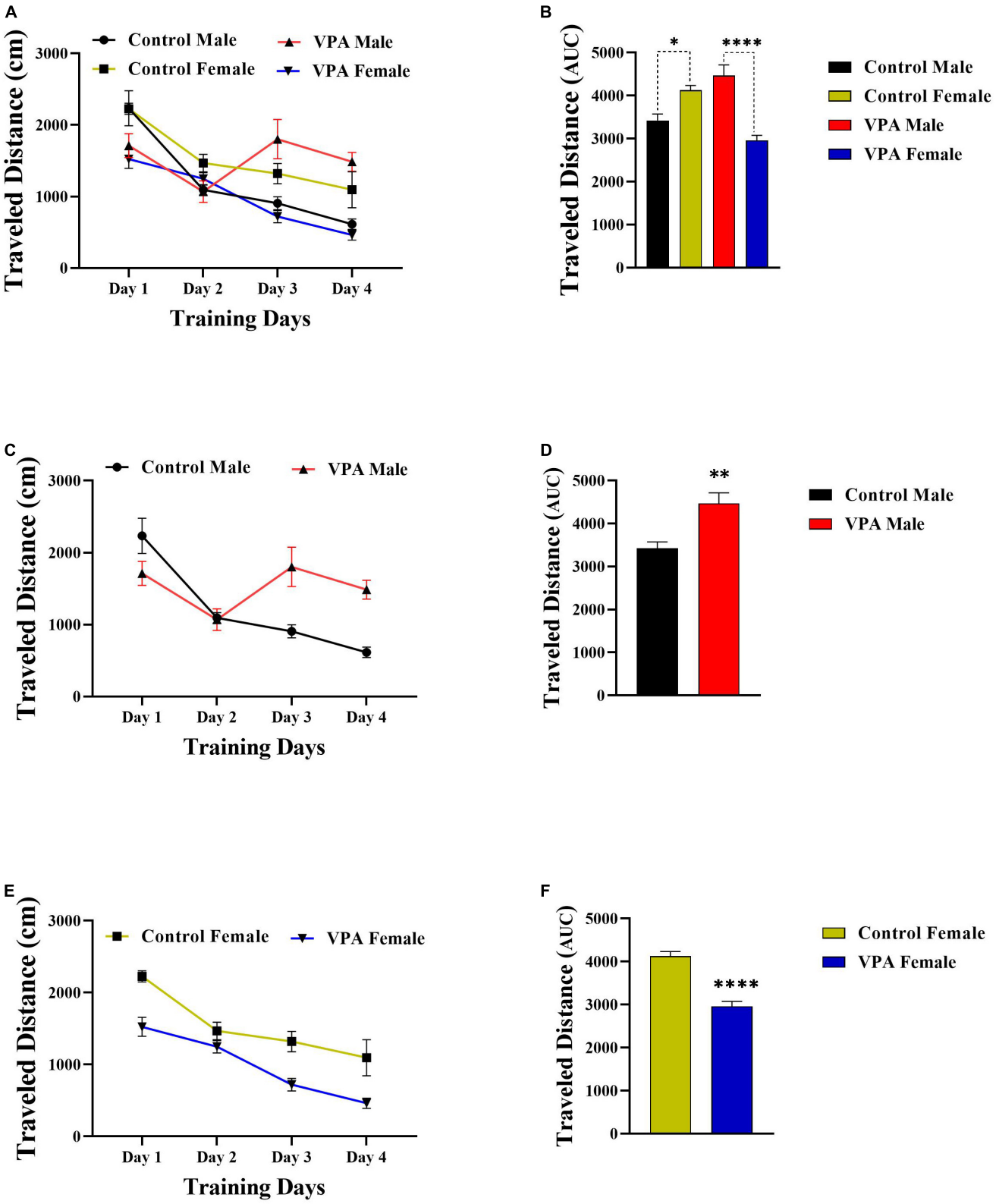

FIGURE 3 | Traveled distance to find the hidden platform during training days. **(A)** Significant sex differences in the distance traveled in control and VPA-exposed male and female offspring. **(B)** AUC for traveled distance. **(C)** Significant differences in the traveled distance in control and VPA-exposed male offspring. **(D)** AUC for traveled distance in male offsprings. **(E,F)** Same analysis as **(C,D)**, respectively, but for female offsprings. Data presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

RESULTS

Sex Difference in the Effects of Prenatal Valproic Acid Exposure on the Morris Water Maze Acquisition in the Male and Female Offspring

As there were significant gender differences in the MWM acquisition in control and VPA-exposed male and female offspring (**Figures 2A,B**, **3A,B**), the data were described separately for male and female offsprings. Overall, in MWM task, male control rats outperformed female control rats. But, VPA-exposed female offspring had a better performance than VPA-exposed male offspring.

Valproic acid-exposed male offspring had significantly shorter swim distance and escape latency to find the hidden platform (during training days) than the male control group. Two-way repeated-measures ANOVA revealed significant effect of day effect [$F(3,53) = 5.476$, $p = 0.0024$], significant effect of VPA [$F(1,53) = 10.56$, $p = 0.0020$], and a significant interaction of the two [$F(3,53) = 3.098$, $p = 0.0344$] in escape latency during the training days in male offspring (**Figure 2C**). Next, the AUC of the escape latency over 4 consecutive days was calculated for each group for statistical comparison. Escape latency to find hidden platform increased in VPA-exposed male offspring animals when compared with male control rats ($p = 0.0022$, **Figure 2D**). In addition, VPA-exposed male offspring traveled more distance than male control rats {day effect [$F(2.449,34.28) = 14.30$, $p < 0.0001$], VPA effect [$F(1,14) = 5.528$, $p = 0.0339$], and interaction of the two [$F(3,42) = 9.523$, $p < 0.0001$], Two-way RM ANOVA, **Figure 3C**}. The AUC of traveled distance was calculated for each group. Distance traveled to find the hidden platform increased in VPA-exposed male offspring animals when compared with male control rats ($p = 0.0027$, **Figure 3D**).

Interestingly, unlike VPA-exposed male offspring, VPA-exposed female offspring had a better performance in MWM training days when compared with the female control group. VPA-exposed female offspring had shorter swimming paths to escape onto the hidden platform, indicating that they had better performance than the female control group. Two-way repeated-measures ANOVA revealed significant effect of day [$F(3,56) = 5.709$, $p = 0.0018$], significant effect of VPA [$F(1,56) = 12.11$, $p = 0.0010$], and a significant interaction of the two [$F(3,56) = 3.964$, $p = 0.0124$] in escape latency during the training days in female offspring (**Figure 2E**). Next, the AUC of the escape latency over 4 consecutive days was calculated for each group. Escape latency to find the hidden platform decreased in VPA-exposed female offspring animals when compared with female control rats ($p = 0.0010$, **Figure 2F**). Similarly, VPA-exposed female offspring traveled less distance than female control rats {day effect [$F(2.100,29.40) = 49.14$, $p < 0.0001$], VPA effect [$F(1,14) = 13.46$, $p = 0.0025$], and interaction of the two [$F(3,42) = 2.548$, $p = 0.0687$], two-way RM ANOVA, **Figure 3E**}. The AUC of traveled distance was calculated for each group. Traveled distance to find the hidden platform decreased in VPA-exposed female offspring animals when compared with female control rats ($p < 0.0001$, **Figure 3F**).

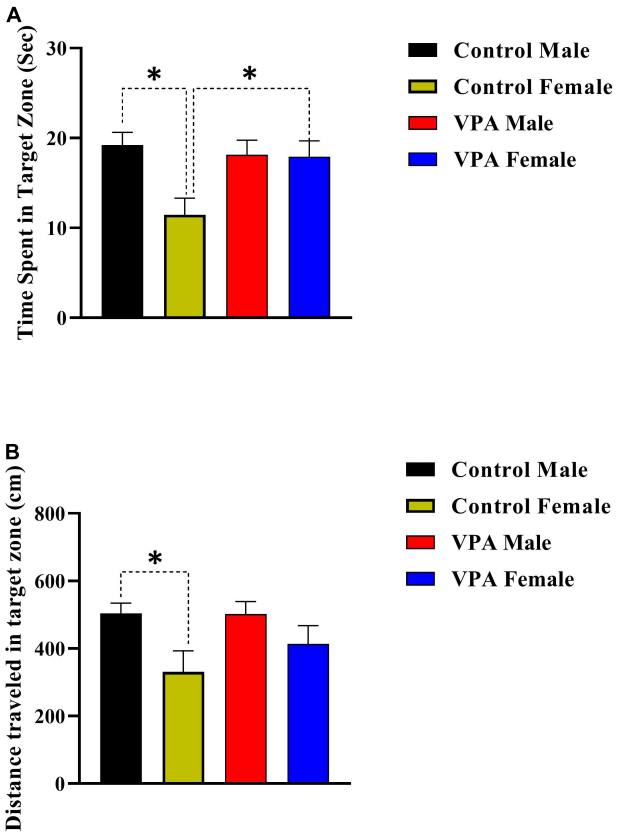

FIGURE 4 | Probe trial performance of male and female rats. There was significant gender difference between male and female control rats but not for VPA-exposed male and female offspring for time spent time **(A)** and traveled distance **(B)** in the target zone. Data are presented as mean ± SEM. *$p < 0.05$.

Male and Female Performance in the Morris Water Maze Probe Trial Reference Memory

The probe trial reference memory test was conducted 24 h after the last training trial. During this test, the platform was removed and time spent and distance traveled in target zone were recorded. There was significant gender difference between male and female control rats but not for VPA-exposed male and female offspring (**Figures 4A,B**), thus the data were described separately for male and for female offsprings. Control female rats spent less time in the target zone in comparison with control male animals ($p < 0.05$, **Figure 4A**). However, there was no significant difference between VPA-exposed male and female offspring ($p > 0.05$, **Figure 4A**). VPA-exposed female offspring animals spent more time in the target zone when compared with female control rats. Time spent in the target zone was almost same for VPA-exposed male offspring and control male animals (**Figure 4A**).

The data for distance traveled in target zone are shown in **Figure 4B**. Control female rats traveled less distance in the target zone in comparison with control male animals (**Figure 4B**).

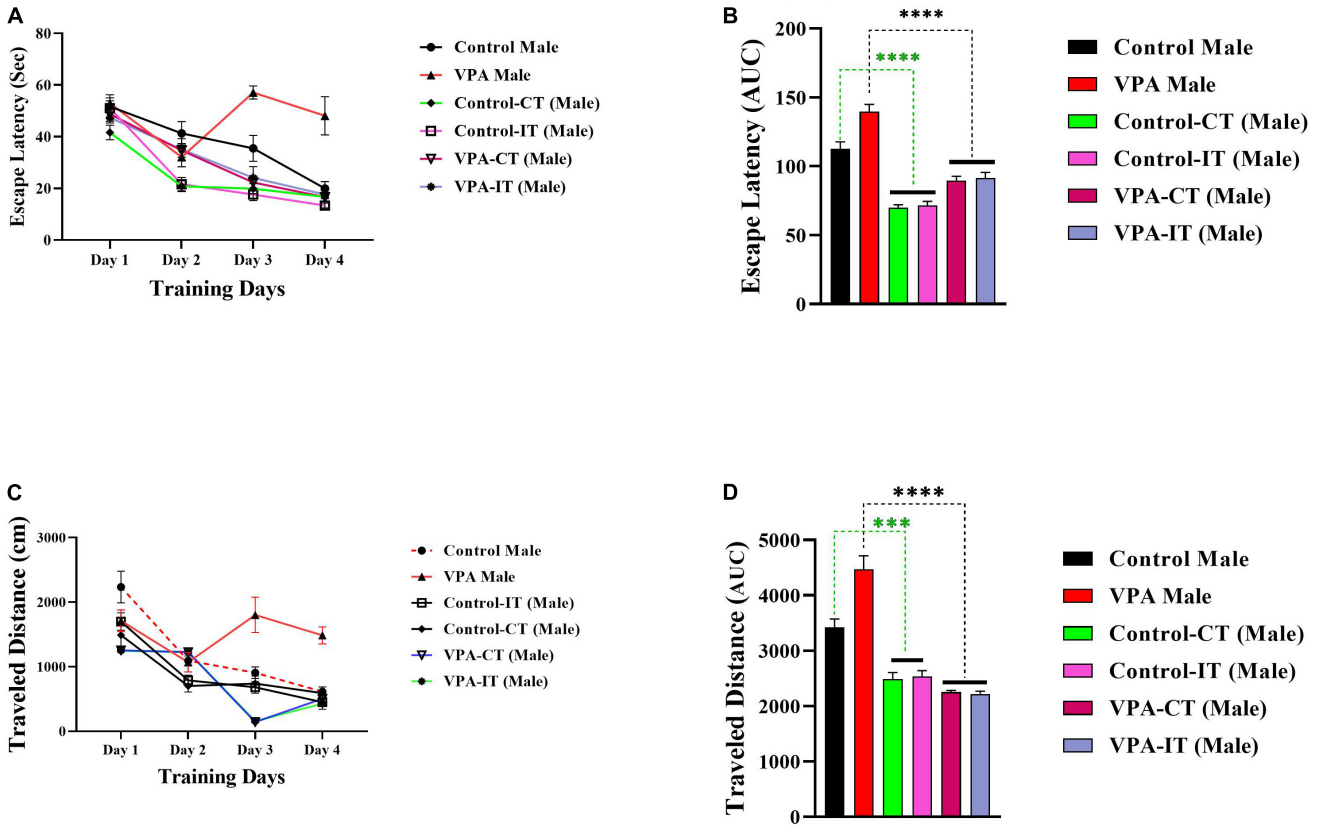

FIGURE 5 | The effects of IT or CT on the MWM acquisition in the control and VPA-exposed male offspring. IT and CT exercise trainings in both control and VPA-exposed male rats significantly reduced the escape latency **(A)** and traveled distance **(C)**. AUC for escape latency **(B)** traveled distance **(D)** in male rats. Data are presented as mean ± SEM. ***$p < 0.001$, ****$p < 0.0001$.

However, there was no significant difference between VPA-exposed male and female offspring (**Figure 4B**). Moreover, there was no significant difference between VPA-exposed male and female offspring in comparison with male and female control rats, respectively (**Figure 4B**).

The Effect of Exercise Interventions on the Morris Water Maze Acquisition in the Control and Valproic Acid-Exposed Male Offspring

The data for the platform location latency and traveled distance in exercise-trained male animals are shown in **Figure 5**. There were significant differences between experimental groups [escape latency: day effect; $F(2.822,107.2) = 82.57$, $p < 0.0001$, exercise effect: $F(5,38) = 15.66$, $p < 0.0001$, interaction; $F(15,114) = 7.085$, $p < 0.0001$, and traveled distance: day effect; $F(3,114) = 78.80$, $p < 0.0001$, exercise effect: $F(5,38) = 21.24$, $p < 0.0001$, interaction; $F(15,114) = 9.558$, $p < 0.0001$, two-way ANOVA, **Figures 5A,C**, respectively]. The AUC of the escape latency (**Figure 5B**) and traveled distance (**Figure 5D**) was calculated for each group. The Bonferroni multiple comparison test and AUC analysis showed that both IT and CT exercise trainings in both control and VPA-exposed male rats significantly reduced the escape latency and traveled distance.

The Effect of Exercise Interventions on the Morris Water Maze Acquisition in the Control and Valproic Acid-Exposed Female Offspring

The data for the platform location latency and traveled distance in exercise-trained female animals are shown in **Figure 6**. There were significant differences between experimental groups [escape latency: day effect; $F(2.774,110.9) = 84.14$, $p < 0.0001$, exercise effect: $F(5,40) = 3.555$, $p = 0.0094$, interaction; $F(15,120) = 2.248$, $p = 0.0081$, and traveled distance: day effect; $F(2.871,114.8) = 89.31$, $p < 0.0001$, exercise effect: $F(5,40) = 11.51$, $p < 0.0001$, interaction; $F(15,120) = 4.065$, $p < 0.0001$, two-way ANOVA, **Figures 6A,C**, respectively]. The AUC of the escape latency (**Figure 6B**) and traveled distance (**Figure 6D**) was calculated for each group. The Bonferroni multiple comparison test and AUC analysis showed that both IT and CT exercise trainings significantly reduced the escape latency and traveled distance in control female rats ($p < 0.0001$). Moreover, IT exercise training (but not CT) significantly reduced

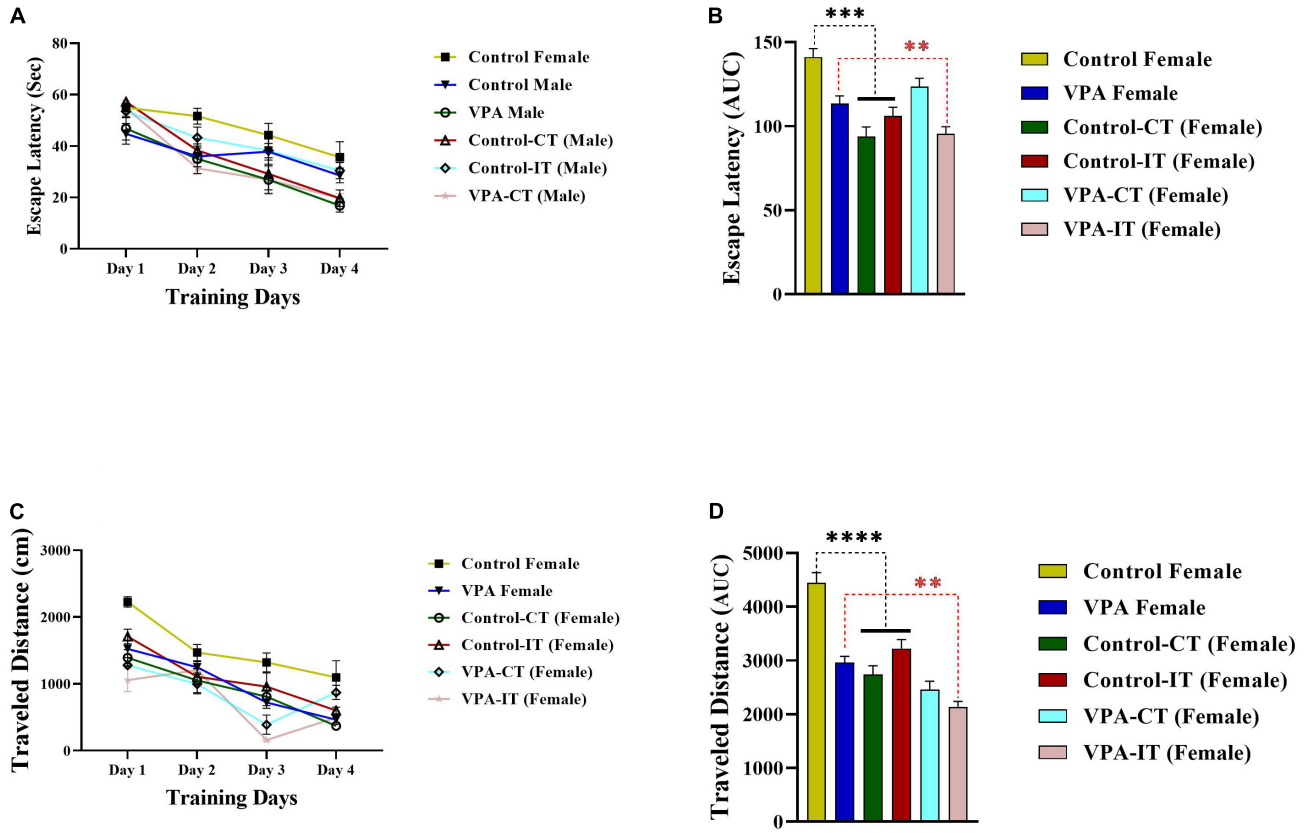

FIGURE 6 | The effects of IT or CT on the MWM acquisition in the control and VPA-exposed female offspring. Both IT and CT exercise trainings significantly reduced the escape latency **(A)** and traveled distance **(C)** in control female rats. IT exercise training (but not CT) reduced the escape latency and traveled distance in VPA-exposed female rats. AUC for escape latency **(B)** traveled distance **(D)** in female rats. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

the escape latency and traveled distance in VPA-exposed female rats ($p < 0.01$).

The Effect of Exercise Interventions on the Morris Water Maze Probe Trial Reference Memory in the Control and Valproic Acid-Exposed Male and Female Offspring

One-way ANOVA followed by Tukey's *post hoc* analysis for male rats showed that both IT and CT exercise trainings in VPA-exposed male rats significantly increased the time spent in the target zone {[$F(5,35) = 3.319$, $p = 0.0148$], **Figure 7A**}. In addition, our results showed that CT exercise training (but not IT exercise training) increased distance traveled in the target zone {[$F(5,35) = 5.328$, $p = 0.0012$], **Figure 7B**}.

In female rats, that both IT and CT exercise trainings in VPA-exposed male rats significantly increased the time spent {[$F(5,37) = 7.660$, $p < 0.0001$], **Figure 7C**} and traveled distance {[$F(5,37) = 8.450$, $p < 0.0001$], **Figure 7D**} in the target zone. Additionally, the swimming speed was the same in all rats, which indicates that there is no motor disorder in these animals (data not shown). In addition, in the visual test, all the rats were able to find the non-hidden platform in less than 10 s, indicating that the animals had no visual problems.

DISCUSSION

A possible beneficial role of exercise interventions and sex differences in spatial learning and memory in prenatal VPA-induced rat model of autism was investigated in the current study. Our observation demonstrated that prenatal exposure to VPA affects the spatial learning and memory in a sex dependent manner. Overall, in MWM acquisition, male control rats outperformed female control rats. However, VPA-exposed female offspring had a better performance than VPA-exposed male offspring. In the case of MWM reference memory, we did not observe a sex difference between VPA-exposed male and female offspring. However, here and in our previous study (Safari et al., 2021), we saw a sex difference between male and female control rats. Both IT and CT exercises in both control and VPA-exposed male rats significantly improved MWM acquisition. Moreover, both IT and CT exercises significantly improved MWM acquisition in control female rats. In addition, IT exercise (but not CT) significantly improved MWM acquisition in

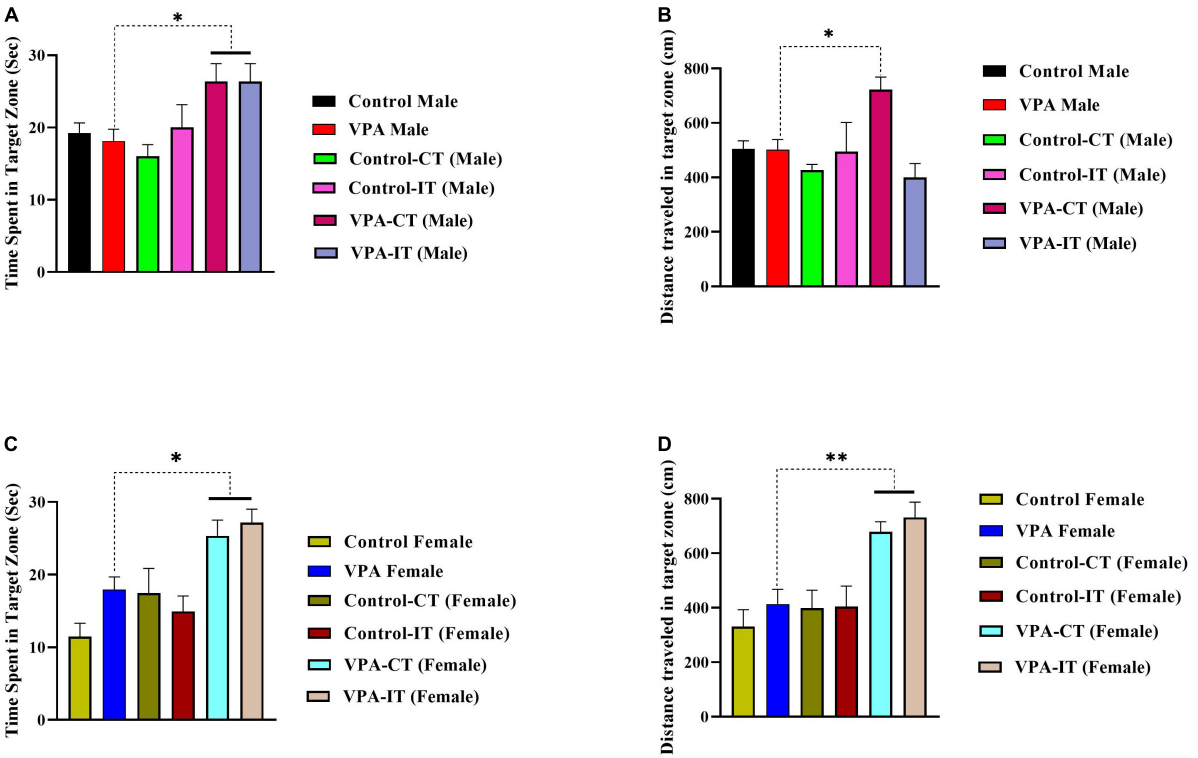

FIGURE 7 | The effects of IT or CT on the MWM probe trial reference memory in the control and VPA-exposed male and female offspring. Both IT and CT exercise trainings in VPA-exposed male rats increased the time spent in the target zone **(A)**. CT exercise training increased distance traveled in the target zone **(B)**. In female rats, that both IT and CT exercise trainings in VPA-exposed male rats increased the time spent **(C)** and traveled distance **(D)** in the target zone. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

VPA-exposed female offsprings. Both IT and CT exercises in VPA-exposed male and female offsprings improved the MWM reference memory.

In our previous work (Safari et al., 2021), we discussed the underlying mechanisms for sex differences in male and female control animals (male control rats outperformed female control rats). However, here we found that VPA-exposed female offspring had a better performance than VPA-exposed male offspring (unlike control animals where male offsprings performed better). Most studies using the VPA model have mainly examined the male sex and sex differences in autistic-like models were described only in few works. Similarly in humans, male and female with autism show different phenotypes. Male mice exposed to VPA showed social impairment and decreased social interaction, which was revealed by the lack of preference for stranger mouse in the three-chamber social experiment (Schneider et al., 2008; Kazlauskas et al., 2019; Ornoy et al., 2019). On the other hand, an increase in repetitive and anxiety-like behaviors has been reported in male and female animals (Ornoy et al., 2019; Sailer et al., 2019). In addition, female animals usually had fewer social and communication problems and fewer repetitive behaviors than male animals (Szatmari et al., 2012; Van Wijngaarden-Cremers et al., 2014; Beggiato et al., 2017). It has been shown that male animals with ASD show poorer perceptual attention to detail (Lai et al., 2012). On the contrary, male animals with ASD performed better in block design performance, while female animals with ASD performed better in the trail making test (Bölte et al., 2011). Edalatmanesh et al. (2013) reported an increase in hippocampal cell density and increased spatial memory in the VPA rat model of autism, but did not examine (or perhaps did not observe) sex differences.

Proposed mechanisms by which VPA exposure during pregnancy causes autistic-like behaviors in both human and rodent offspring are as follow (Ornoy, 2009; Mabunga et al., 2015; Taleb et al., 2021): excitation-inhibition (E:I) imbalance, brain inflammatory challenges, reduction of neurogenesis, oxidative stress, changes in serotonergic, dopaminergic and/or oxytocinergic systems, folic acid deficiency, effects on Wingless and Int-1 (Wnt) signaling, changes in gamma-aminobutyric acid (GABA) and serotonin homeostasis and activity, alteration in neuronal spine density, and changes in calcium/calmodulin-dependent protein kinase II (CaMKII)/protein kinase A (PKA)/protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR), glycogen synthase kinase-3β (GSK-3β)/β-catenin signaling pathways. In addition, VPA directly inhibits histone deacetylase (HDAC), causing transient hyperacetylation in the brain (Phiel et al., 2001; Zimmermann et al., 2015).

The number of research has shown that the transcriptional levels of related genes, enzymes, and membrane proteins are

likely to be affected by VPA, leading to ASD (Weinstein-Fudim et al., 2019; Taleb et al., 2021). So far, genetic studies identified more than 1,000 genes that contribute to ASD risk (Wiśniowiecka-Kowalnik and Nowakowska, 2019). Behavioral differences and other sex differences in autism may be related to genes that are affected by VPA differently in male and female animals (Weinstein-Fudim et al., 2019). Gender differences in gene expression involved in glutamatergic pathways (such as Unc13a and Cacna1a) may explain sex differences in ASD pathology (Lipstein et al., 2017; Weinstein-Fudim et al., 2019). It is clear that glutamate is involved in learning and memory.

Our present study revealed that both IT and CT exercise trainings improved the spatial learning and memory in the autistic and non-autistic rats. Thus, these results suggest that both CT and IT have beneficial effects on spatial memory. The underlying mechanisms of exercise-induced memory improvement in autistic and healthy rats can be discussed as follows.

Seo et al. (2013) has shown that VPA exposure decreases the expressions of PI3K, p-Akt, and p-ERK1/2 in the hippocampus, and treadmill exercise increases the neurogenesis and the expressions of reelin and its down-stream molecules (PI3K, p-Akt, and p-ERK1/2) in the hippocampus of the autistic rats. They conclude that treadmill exercise improves the spatial memory through the activation of reeling signaling pathway in the VPA-induced autistic rats. In addition, Park et al. (2021) reported that maternal swimming exercise during pregnancy improves memory function by increasing the cell proliferation and inhibiting apoptosis through Wnt/β-catenin signaling cascade activation in the VPA injected pups.

Mice exposed to VPA showed the lower levels of cortical expression of brain-derived neurotrophic factor (BDNF) mRNA (Roullet et al., 2010). It is clear that BDNF is necessary and sufficient for memory (Bekinschtein et al., 2008). Meanwhile, maternal swimming exercise during pregnancy effectively enhanced the BDNF level in the VPA injected pups (Park et al., 2021). Additionally, they showed that maternal swimming exercise inhibited β-catenin, Bcl-2 related X protein (Bax) and cleaved caspase-3 expression and enhanced B-cell lymphoma 2 (Bcl-2) expression in the VPA injected pups (Park et al., 2021).

In addition to the above and based on the literature, the benefits of physical exercise on learning and memory in autistic and healthy rats seem to be mediated by several mechanisms, such as: the production of neurotrophic factors, myelin protection, increased cell proliferation, neurogenesis and plasticity, reduced neuron death, decrease in oxidative stress, and neuroinflammation (Phillips et al., 2014).

Some neurodevelopmental disorders, such as autism, are quite limited to humans. Besides, there are many limitations and precautions in designing rodent models. The most important one is that animals cannot replicate all of the uniquely human components of autism. In conclusion, our observation demonstrated that prenatal exposure to VPA affects the spatial learning and memory in a sex dependent manner. We have shown that both IT and CT exercises are able to improve the cognitive function in healthy and autistic rat pups. Since the nervous system controls the cognitive behavior, these sex-related functional differences may be associated to the sex-specific structure of the neuronal circuits in the nervous system (Cosgrove et al., 2007). Future studies are needed to validate our findings in humans ASD, such as behavioral testing across sex.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Study Ethics Committee of Hamadan University of Medical Sciences.

AUTHOR CONTRIBUTIONS

SK designed the project, wrote the manuscript, performed the statistical analysis, revised the manuscript, and supervised the project. RG, RM, and IS were involved in laboratory works and experimental design of the work. MZ was involved in laboratory assessments. All authors read and approved the final results.

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Regular Low-Intensity Exercise Prevents Cognitive Decline and a Depressive-Like State Induced by Physical Inactivity in Mice: A New Physical Inactivity Experiment Model

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Regular exercise has already been established as a vital strategy for maintaining physical health *via* experimental results in humans and animals. In addition, numerous human studies have reported that physical inactivity is a primary factor that causes obesity, muscle atrophy, metabolic diseases, and deterioration in cognitive function and mental health. Regardless, an established animal experimental method to examine the effect of physical inactivity on physiological, biochemical, and neuroscientific parameters is yet to be reported. In this study, we made a new housing cage, named as the physical inactivity (PI) cage, to investigate the effect of physical inactivity on cognitive function and depressive-like states in mice and obtained the following experimental results by its use. We first compared the daily physical activity of mice housed in the PI and standard cages using the nano-tag method. The mice's physical activity levels in the PI cage decreased to approximately half of that in the mice housed in the standard cage. Second, we examined whether housing in the PI cage affected plasma corticosterone concentration. The plasma corticosterone concentration did not alter before, 1 week, or 10 weeks after housing. Third, we investigated whether housing in the PI cage for 10 weeks affected cognitive function and depressive behavior. Housing in an inactive state caused a cognitive decline and depressive state in the mice without increasing body weight and plasma corticosterone. Finally, we examined the effect of regular low-intensity exercise on cognitive function and depressive state in the mice housed in the PI cage. Physical inactivity decreased neuronal cell proliferation, blood vessel density, and gene expressions of vascular endothelial growth factors and brain-derived neurotrophic factors in the hippocampus. In addition, regular low-intensity exercise, 30 min of treadmill running at a 5–15 m/min treadmill speed 3 days per week, prevented cognitive decline and the onset of a depressive-like state caused by physical inactivity. These results showed that our novel physical inactivity model, housing the mice in the PI cage, would be an adequate and valuable experimental method for examining the effect of physical inactivity on cognitive function and a depressive-like state.

Keywords: physical inactivity, low-intensity exercise, cognitive function, depressive-like state, hippocampus

INTRODUCTION

Regular exercise has already been established as a vital strategy for maintaining physical health in humans and animals. Numerous human studies have reported that physical inactivity is a primary factor that causes obesity, muscle atrophy, and metabolic diseases (Booth et al., 2017; Bowden Davies et al., 2019). Moreover, physical inactivity (lack of exercise) is the fourth leading risk factor for death in the world after hypertension, diabetes, and smoking (Lee et al., 2012) and is linked to decreased cognitive function and a decline in mental health in older adults (Hamer and Stamatakis, 2014; Cunningham et al., 2020). In recent years, the increase in working from home and the suspension of sports facilities due to the infectious spread of the coronavirus disease (COVID-19) is increasing the number of patients with diseases related to physical inactivity worldwide. There have been only a few studies, however, investigating the mechanism underlying cognitive decline due to physical inactivity and the preventive effects of exercise against physical inactivity-induced cognitive decline.

Many previous studies investigating physical inactivity using animals targeted the muscle atrophy induced by the disuse of skeletal muscles rather than the lack of physical activity; in these studies, hindlimb suspension (Theilen et al., 2018), Gibbs fixation (Morimoto et al., 2013; Ye et al., 2013) and hindlimb nerve denervation (Lala-Tabbert et al., 2019) were used as the experimental methods for causing skeletal muscle atrophy. Such an experimental method can, indeed, cause skeletal muscle atrophy. It is important to note that these methods generally cause severe stress in animals and are not suitable for examining the effect of long-term physical inactivity on cognitive function and depressive states because stress usually leads to cognitive decline and depressive states.

Recently, two new experimental models have been reported to examine the physiological influence of physical inactivity. In one model, the investigators analyzed mice housed in a smaller cage than the standard cage that prevented climbing on the cage lid (Roemers et al., 2019). In another model, mice trained with a running wheel were subsequently denied exercise by removal of the running wheel (Nishijima et al., 2013). However, experimental conditions that induce physical inactivity in animals could simultaneously bring about isolation stress for animals. Furthermore, isolation stress causes cognitive decline (Rivera et al., 2020) and depressive disorder (Lee et al., 2020) in experimental animals. Therefore, isolation stress needs to be avoided to accurately examine the effect of physical inactivity on cognitive function and depressive disorder. With this in mind, the previous two studies did not take measures to avoid isolation stress.

Adult hippocampal neurogenesis is a phenomenon in which neurons are newly generated in the hippocampal dentate gyrus; the rate of hippocampal neurogenesis is decreased by aging (Yang et al., 2015; Horowitz et al., 2020) and chronic stress (Kiuchi et al., 2012; Bambico et al., 2015; Planchez et al., 2021) and is increased by regular exercise (Kiuchi et al., 2012; Choi et al., 2018) and antidepressant drugs (Jedynak et al., 2014). Furthermore, previous studies have shown that adult hippocampal neurogenesis occurs near the local microvasculature of the hippocampus (So et al., 2017) and that chronically stressed mice exhibit decreased capillary density in the hippocampal dentate gyrus (Kiuchi et al., 2012). Regarding the relationship between neurogenesis and blood vessels in the hippocampus, Fournier and Duman (2012) demonstrated that stress-induced decrease in hippocampal neurogenesis mainly occurred near capillaries and suggested that decreased blood flow due to chronic stress led to blood flow suppression and decreased capillary density in the hippocampus, followed by depression. These findings suggest that the development and improvement of a depressive state may be closely related to changes in neurogenesis and angiogenesis in the hippocampus. Moreover, vascular endothelial growth factor (VEGF) is critical in hippocampal neurogenesis and angiogenesis (Rich et al., 2017). A previous study showed that neurogenesis and angiogenesis were enhanced by antidepressant drugs and VEGF overexpression (Udo et al., 2008), whereas VEGF receptor inhibitors canceled the effect of antidepressant drugs (Warner-Schmidt and Duman, 2007; Greene et al., 2009), thus indicating that VEGF is necessary for neurogenesis, angiogenesis, cognitive maintenance, and antidepressant action. The effects of physical inactivity on adult hippocampal neurogenesis, angiogenesis, and VEGF have not yet been elucidated.

Here, we made a unique new cage, named as the physical inactivity (PI) cage using transparent acrylic plates to decrease the mice's physical activity while reducing isolation stress as much as possible. Using the PI cages, we compared the amount of physical activity of the mice housed in the PI cages and the standard cages, examined the effect of physical inactivity on cognitive function and a depressive-like state, and analyzed the preventative effect of regular low-intensity exercise on behavioral alteration due to physical inactivity.

MATERIALS AND METHODS

Animal and Ethical Approval

We conducted all experimental procedures and animal treatments according to the laboratory animal manual guidelines of Nippon Medical School. This study was approved by the Animal Care and Use Committee of Nippon Medical School (approval number was 28-023) and complies with animal research (ARRIVE) guidelines. Male C57BL/6J mice (10 weeks old, $n = 73$) were purchased from Sankyo Lab Service Co. (Tokyo, Japan) and used for all experiments. All mice were housed under 23 ± 2℃ and with a 12-h light/dark cycle (light on 08:00–20:00). Standard chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were supplied *ad libitum*.

Inactivity Cage

Previous studies have shown that the mice's regular cage (33 cm length, 22 cm height) was partitioned into six parts with aluminum boards to decrease the mice's physical activity (Nakajima et al., 2009, 2010). For the present study, we made new cages to decrease the physical activity of the mice using acrylic plates and called the structure the physical inactivity

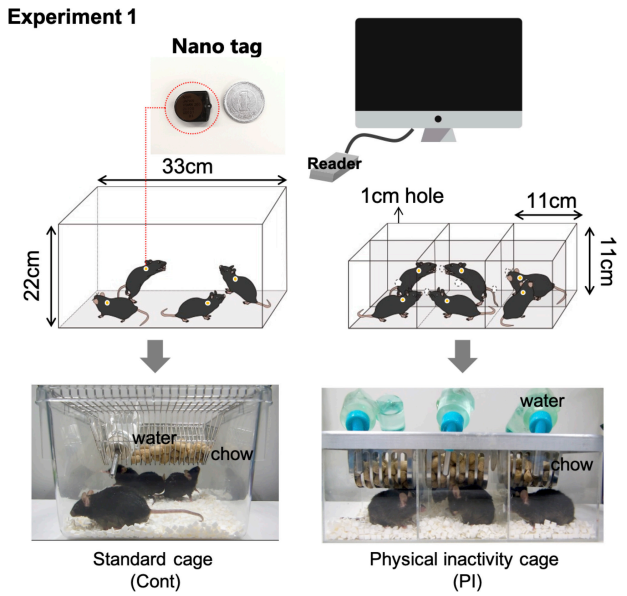

FIGURE 1 | Measurement of the daily physical activity of mice using nano-tag apparatuses. Graphical explanation of measurement of the daily physical activity of mice using the nano-tag method.

(PI) cage (**Figure 1A**). In the PI cage, the residential space for a mouse was 11 cm in length and 11 cm in height, equal to that in the aluminum-divided cage; however, the PI cage differed from the aluminum-divided cage in the following two points. First, each mouse could visually see the mouse in the next compartment, as the cage was built using a transparent acrylic plate. Second, the mice could touch each other's noses through a 12-mm hole drilled in the wall (**Figure 1**). When the mice are bred individually, they are usually exposed to isolation stress, which is caused by perturbing social interaction (Arzate-Mejia et al., 2020). Perturbation of social interaction would be caused by being visually isolated from other mice and losing physical contact with the other mice. Therefore, the PI cage was modified in two manners shown above. The housing contained food and water, so the mice could freely consume chow and tap water from the bait box and water bottle mounted on the cage ceiling, respectively.

Experimental Design

In the present study, we conducted the following four experiments. In experiment 1, we compared the physical activity of the mice housed in the PI cage and the standard mouse cage using a nano-tag (**Figures 1**, **2**). Experiment 2 investigated whether housing mice in the PI cage affected their plasma corticosterone concentration (**Figure 3**). Experiment 3 investigated whether housing the mice in the PI cage for 10 weeks affected their cognitive function and depressive behaviors (**Figure 4**). Finally, experiment 4 investigated the effect of regular low-intensity exercise on cognitive function and depressive state in the mice housed in the PI cage for 20 weeks (**Figure 5**).

Experiment 1

Physical Activity Measurement

We compared the locomotor activity of the mice housed in the PI cage and standard mouse cage (**Figure 1**). For this purpose, we implanted nano-tags onto the back skin of the mice under anesthesia. Nano-tags (KISSEI COMTEC, Matsumoto, Japan) can be surgically implanted in a small laboratory animal body and quantify physical activity by measuring frequency and amount of vibration using a three-dimensional accelerometer inside them and recording the data in the device's internal memory (Sakai et al., 2020). After a week's recovery from surgery, the physical activity levels of the mice measured by the implanted nano-tag were recorded in the PI or the standard mouse cages for two consecutive days, respectively (**Figure 1**). Using nano-tags allowed locomotive levels in the standard cage to be measured when the subject mouse was residing with the other mice.

Experiment 2

Examination of Plasma Corticosterone During Long-Term Housing in the Physical Inactivity Cages

Mice were housed in the PI cage for 10 weeks, and blood was collected from a tail vein under gas anesthesia with isoflurane using a small animal anesthesia device (SN-487-0T; Sinano Mfg. Co., Ltd., Tokyo, Japan) (**Figure 3A**). Blood was collected before housing and at the first and tenth weeks of housing. Subsequently, blood was centrifuged to collect plasma which was stored at –80°C until analysis.

Experiment 3

Long-Term Housing in the Physical Inactivity Cages

Twenty-two mice were divided into control (Cont) mice and physical inactivity (PI) mice groups. In the former group, four mice were housed in each standard mouse cage, whereas in the latter, one mouse was housed in each compartment of the PI cage. Under each condition, the mice were housed for 10 weeks, then subjected to several behavioral tests to examine their cognitive function and depressive state. Two days after the last session of behavioral testing in Experiment 3, we dissected all the mice between 10 a.m. and 3 p.m. (**Figure 4A**). Plasma was isolated and stored at –80°C as described above.

Experiment 4

Long-Term Housing in the Physical Inactivity Cages With Regular Low-Intensity Exercise

Thirty-three mice were divided into three groups. (1) Control (Cont) mice: Four mice per cage were housed in a standard mouse cage. (2) Physical inactivity (PI) mice: housed in the PI cage. (3) Physical inactivity and exercised (PI + Ex) mice: housed in the PI cage and subjected to 30 min of treadmill running at a treadmill speed of 5–15 m/min, 3 days per week (**Figure 5A**). The treadmill speed was changed depending on the physical conditions of the mice on the exercise day. Twenty weeks later, all mice were examined for muscle strength, endurance exercise capacity, and behavioral tests to analyze the cognitive function and depressive state. On days two and three after the last session of behavioral tests in Experiment 4, we dissected all the mice between 10 a.m. and 3 p.m. as follows. First, the

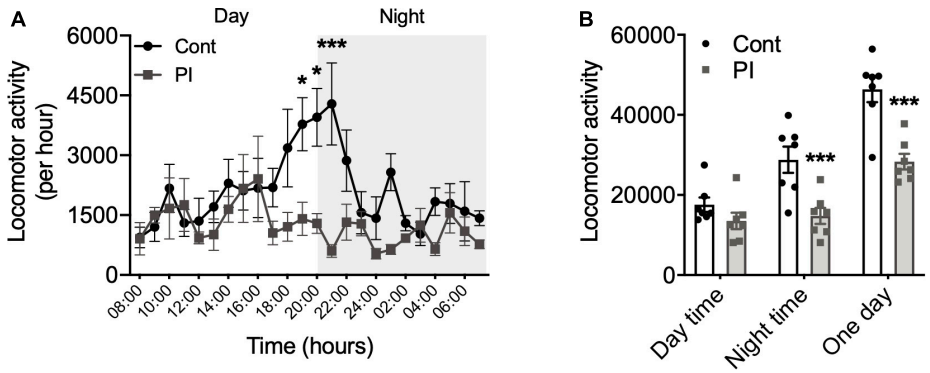

FIGURE 2 | Comparison of the physical activity amount in the standard and PI cages. **(A)** Physical activity amount per hour during 1 day. **(B)** Physical activity amount during daytime (left), during nighttime (center), during 1 day (right). All data are presented as the mean ± SEM (Cont cage, $n = 7$; PI cage, $n = 6$). Data were analyzed using two-way ANOVA with Bonferroni's *post-hoc* test **(A)**, and unpaired *t*-test analyzed data **(B)**. $^{*}p < 0.05$, $^{***}p < 0.001$ in comparison with the Cont mice.

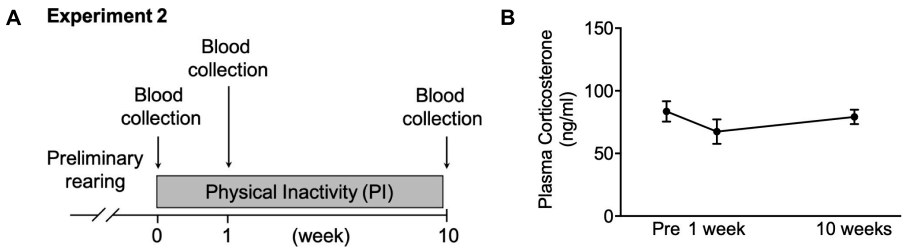

FIGURE 3 | Changes in plasma corticosterone concentration during housing in the PI cage. **(A)** Schedule for collecting blood during housing in the PI cage. **(B)** Plasma corticosterone concentration before housing and at 1st and 10th weeks. All data are presented as the mean ± SEM ($n = 10$). Data were analyzed using one-way ANOVA with Dunnett's *post-hoc* test.

mice were anesthetized by intraperitoneal injection of three mixed anesthesia (containing Domitor, Midazolam, Bettlefar, and saline) at 0.75 mg/kg. Next, body weight was measured, blood was collected *via* heart puncture, and the heart was perfused with saline *via* the right ventral vein. We then collected the brain, epididymal fat, and skeletal muscles, including the soleus, extensor digitorum longus (EDL), and gastrocnemius samples. The brain was cut in half, and the hippocampus was separated from the right hippocampus. The brain's left hemisphere was fixed for histochemical analysis as described below. The collected samples (plasma, hippocampus, soleus, gastrocnemius, EDL, and epididymal fat) were frozen in liquid nitrogen and stored at -80°C until analysis.

Measurement of Muscle Strength and Endurance Exercise Capacity

We used a digital grip strength meter (GPM-100; Melquest, Japan) to measure forelimb or all-limb grip strength, based on the study of Takeshita et al. (2017); the apparatus measured the grip strength of the mice and showed the peak force strength (in grams). To measure the forelimb or all-limb grip strength, we had the mice grasp the device's grip with their forelimb or all limbs and pulled the mouse tail from behind. The tension recorded by the gauge when the mice released their limbs from the bar was measured and expressed as grip strength (in grams). Results of three measurements per mouse were averaged and expressed as grip strength (gram per gram of body weight).

We also conducted a running test on a treadmill to examine the endurance exercise capacity of the mice according to the method of Rowe et al. (2013) with a slight modification. Before the endurance test, the control and PI mice, which did not undergo regular running on the treadmill, were acclimated to the exercise by running on the treadmill with no tilt angle for 10 min, at the speed of 12 m/min, three times a week for 1 week. In the endurance test, using the treadmill with no treadmill tilt angle, the treadmill speed was initially 5 m/min. After 5 min, the speed was subsequently increased by 1 m/min every 1 min to a maximum 15 m/min speed. After maximum speed was reached, the mice ran at the same speed until exhaustion (**Figure 6C**).

Behavioral Tests

Y-Maze Test

We conducted a Y-maze test to examine working memory based on the study of Rubaj et al. (2003) with slight modifications. The Y-maze consisted of three equally spaced arms (height, 12 cm; width, 3 cm; length, 40 cm). Initially placed at the end of one arm, the mice freely traversed the apparatus while video-recorded for 8 min. Complete entry was determined when the mouse's hind paws had entirely entered an arm of the maze. "Right" choice was defined as consecutive entries into the three

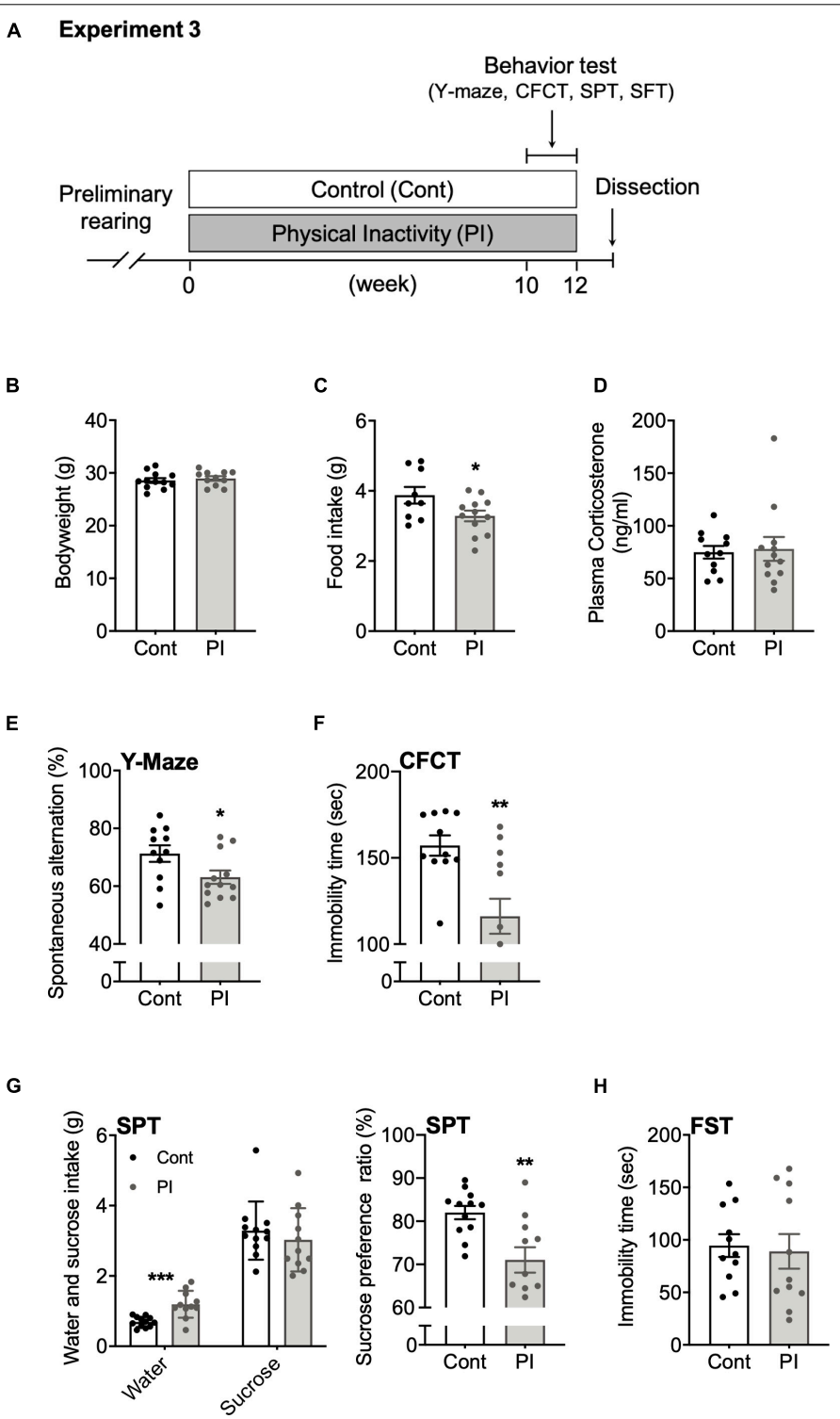

FIGURE 4 | Housing in the PI cage induced cognitive decline and a depression-like state without increasing plasma corticosterone concentration. **(A)** Experimental protocol for physical inactivity housing for 10 weeks and behavioral tests. **(B)** Body weight. **(C)** Food intake on the 10th week. **(D)** Plasma corticosterone concentration. **(E)** Accuracy rate in the Y-maze test. **(F)** Immobility time in the contextual fear conditioning test (CFCT). **(G)** Sucrose preference ratio in the sucrose preference test (SPT). **(H)** Immobility time in the forced swim test (FST). All data are presented as the mean ± SEM (Control mice, $n = 12$; PI mice, $n = 11$). The Student's unpaired t-test analyzed data. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the Cont mice.

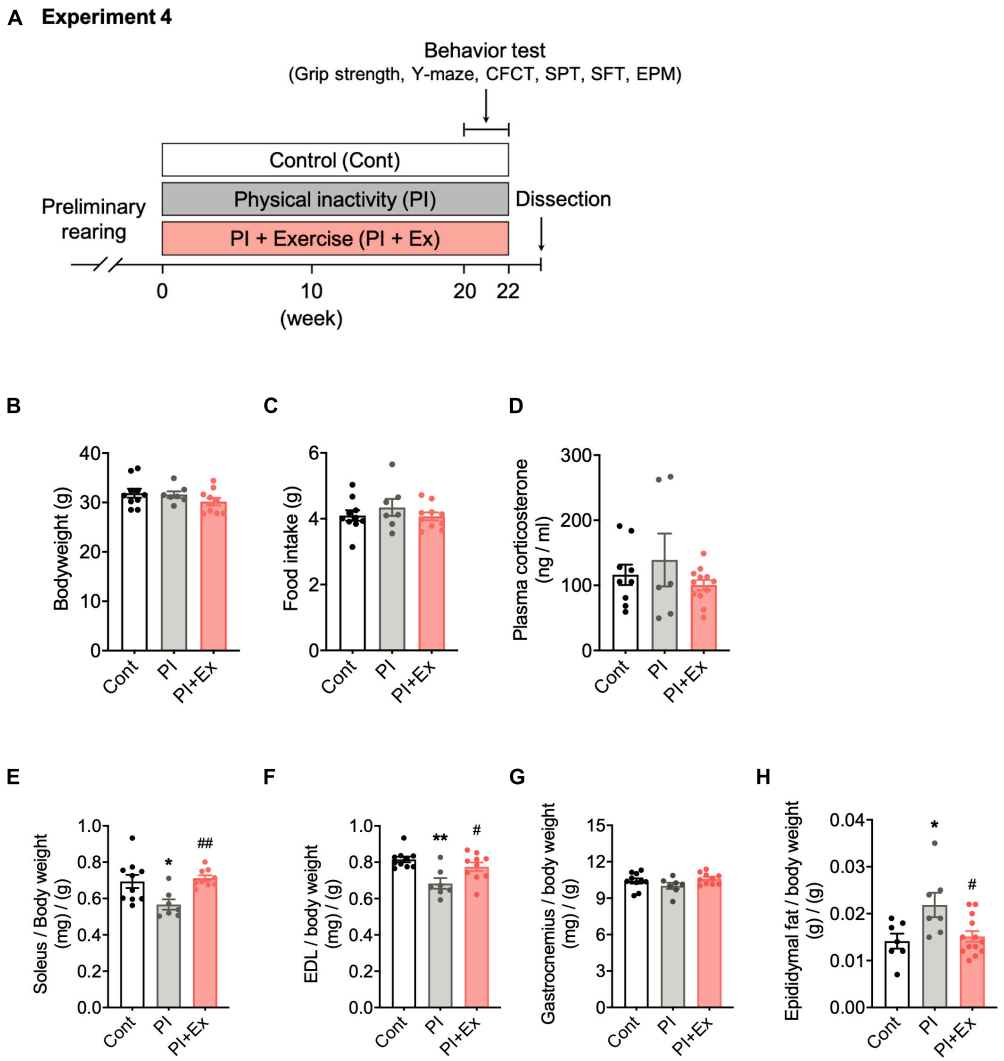

FIGURE 5 | Regular low-intensity exercise prevented muscle weight loss and fat mass gain from housing in the PI cage. **(A)** Experimental design for physical inactivity housing for 20 weeks and cognitive testing. **(B)** Body weight. **(C)** Food intake on 20th week. **(D)** Plasma corticosterone concentration on 20th week. **(E–G)** Soleus, Extensor digitorum longus (EDL), and Gastrocnemius muscle weight normalized to body weight. **(H)** Epididymal fat weight normalized to body weight. All data are presented as the mean ± SEM (Cont mice, $n = 10$; PI mice, $n = 7$; PI + Ex mice, $n = 10$). Data were analyzed using one-way ANOVA with Tukey's *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the Cont mice and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with PI mice.

different arms. Spontaneous alternation was calculated as the percentage of correct entries to the total number of entries using the following formula: percentage alternation (%) = (number of alternations/total number of arm entries) × 100 (%).

Contextual Fear Condition Test

We also performed a slightly modified contextual fear-conditioning test (CFCT), based on the method of Uwaya et al. (2016) to measure long-term memory using the foot shock system model (MK-450MSQ; Muromachi Kikai Co., Ltd., Japan). After being left in the test box for 2 min, the mice received three electric foot shocks (0.8 mA, 2-s duration) with a 2-min interval; they were kept in the test box for an additional minute and were returned to their home cages. On the following day, they were placed in the same test box for 5 min and video-recorded to measure immobility time. Immobility time was analyzed using the Smart 3.0 software (Panlab Inc., Spain).

Sucrose Preference Test

We performed the sucrose preference test (SPT) in the mice's home cages to measure depressive-like state based on the method of Muto et al. (2014). First, all mice were acclimatized to two-bottle conditions for 2 days. Subsequently, the mice were provided with two bottles during nighttime (6 p.m. to 10 a.m.): One contained water, and another 1% sucrose, which allowed them to choose freely. Then, tests were conducted for 3 days, during which the bottle placements (i.e., left and right sides) were interchanged daily. Water and sucrose intake was measured by weighing the bottles before and after the test. Sucrose preference ratio was calculated as follows: Sucrose preference percentage

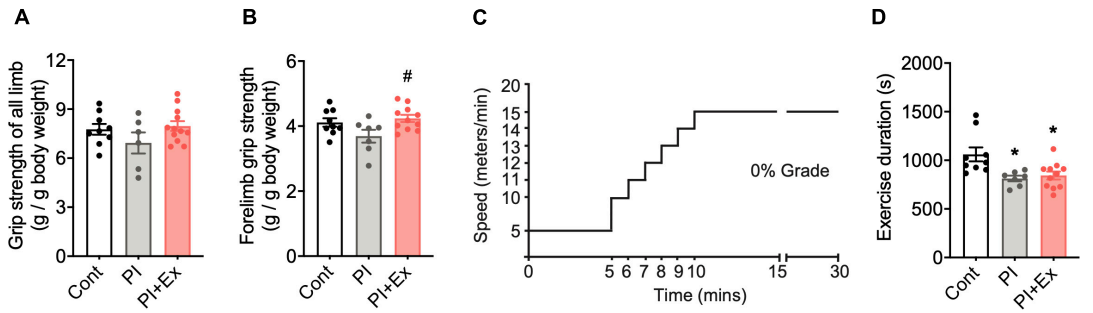

FIGURE 6 | Regular low-intensity exercise prevents decreased muscle strength due to physical inactivity housing but shows no improvement in endurance exercise capacity. **(A,B)** Both all-limb and forelimb grip strengths normalized to body weight. **(C)** Protocol of endurance exercise test. **(D)** Time (seconds) that mice could run for until exhaustion in endurance exercise test on the treadmill. All data are presented as the mean ± SEM (Cont mice, $n = 10$; PI mice, $n = 7$; PI + Ex mice, $n = 10$). Data were analyzed using one-way ANOVA with Tukey's *post-hoc* test. $^{*}p < 0.05$ compared with the Control mice and $^{\#}p < 0.05$ compared with PI mice.

(%) = (volume of sucrose intake/total volume of sucrose and water intake) × 100 (%).

Forced Swimming Test

We performed the forced swimming test (FST) according to Petit-Demouliere et al. (2005). The mice were placed in a water-filled cylinder (height, 20 cm; diameter, 15 cm; water temperature, 25°C) and video recorded for 6 min. In addition, immobility time, determined by the mice's floating duration during the last 4 min of the test, was measured using analytical software (Smart 3.0, Panlab Inc., Spain).

Elevated Plus Maze Test

The elevated plus maze (EPM) was performed according to the method of Moon et al. (2014). The EPM apparatus comprised two open and two closed arms connected to a common central platform. A single pillar, 50 cm in height from the room floor, supported the arms and the central platform. Initially, the mice were placed at the center of the platform and allowed to explore both arms for 5 min. The number of times that the mouse entered each arm, determined by both forefeet entering an arm, and the amount of time they spent in each arm were measured using analytical software (Smart 3.0, Panlab Inc., Spain).

Immunohistochemical Analysis

For the histochemical analysis, the right brain hemispheres were post-fixed in 4% paraformaldehyde at 4°C overnight. The brain was extracted and sectioned as previously described (Kiuchi et al., 2012). Ki-67 sections were incubated for 30 min with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the sections were exposed to heat (100°C) in 100 mM citric acid buffer (pH 6.0) for 30 min using a microwave for antigen retrieval, and the sections were incubated and then blocked with normal goat serum for 2 h. Next, the sections were incubated with primary rabbit polyclonal anti-Ki67 antibody (1:500; Abcam, Cambridge, United Kingdom) with gentle shaking at 4°C for two nights. After washing with PBS, the sections were incubated with goat anti-rabbit biotinylated IgG (1:100; Vector Laboratories, Burlingame, CA) for 2 h. Next, Ki67 sections were incubated with avidin-biotin-horseradish peroxidase complex (VECTASTAIN ABC Kit reagent; Vector Laboratories) for 2 h. Vascularization was examined using CD31 immunohistochemistry. After incubating the sections with 3% hydrogen peroxide for 30 min, and normal rabbit serum for 1 h, they were incubated with an anti-mouse CD31 monoclonal antibody (1:50; BD Pharmingen, CA) for 2 nights at 4°C. After washing with PBS, the sections were incubated with goat anti-rabbit biotinylated IgG (1:100; Vector Laboratories, United States) for 2 h at room temperature. Finally, Ki-67 and CD31 sections were washed with PBS and developed using 3,3′-diaminobenzidine for 2 min. The sections that reacted with antibodies were mounted, dehydrated, and coverslipped using Permount mounting medium. The number of Ki67-positive cells and surface capillary density of CD31 in the hippocampal dentate gyrus were counted using a Leica DM3000 microscope (Leica, Germany). The areas of the hippocampal dentate gyrus were also measured using NIH ImageJ software (NIH Image Engineering, Bethesda, MD, United States) and the cell density per mm^3 calculated.

Isolation of Total RNA and Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction

To measure the mRNA expression of hippocampal brain-derived neurotrophic factor (BDNF), VEGF, the frozen hippocampus and soleus muscle were homogenized on ice in TRIzol lysis reagent (Qiagen, Valencia, CA). Total RNA was extracted from the homogenate according to the manufacturer's instructions. Total RNA was quantified by measuring the absorption at 260 and 260/280 nm ratio to assess concentration and purity. Complementary DNA was synthesized using 1 μg of total RNA in a 20-μl reaction with the ReverTra Ace^{TM} qPCR RT Master Mix with gDNA Remover (FSQ-301; Toyobo, Osaka, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, United States)

to quantify the mRNA levels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers amplified the endogenous control product. The mouse-specific primers used were as follows: VEGF: forward 5′-CGTTTAACTCAAGCTGCCTCGC-3′, reverse 5′-CTTCCAGGAGTACCCCGACGAGATA-3′; BDNF: forward 5′-TGCAGGGGCATAGACAAAAGG-3′, reverse 5′-CTTATGAATCGCCAGCCAATTCTC-3′; GAPDH: forward 5′-CATCACTGCCACCCAGAAGA-3′, reverse 5′-ATG TTCTGGGCAGCC-3′. The 2-ΔΔCT method was used to analyze relative mRNA expression values.

Western Blot

To measure the VEGF content in the skeletal muscle, a portion of the gastrocnemius muscle was homogenized in RIPA lysis buffer [50 mM Tris-HCL buffer (pH 7.4); 150 mM NaCl; 1% Triton X-100; 0.5% Sodium Deoxy Cholate; 0.1% Sodium Dodecyl Sulfate] containing a proteinase inhibitor cocktail (Sigma-Aldrich, MI) and centrifuged at 14,000 × g for 10 min at 4°C. The supernatant's protein concentration was determined using a BCA protein assay kit (Pierce). Aliquots (20 μg protein) were mixed with sodium dodecyl sulfate (SDS) sample buffer containing 1% mercaptoethanol, boiled for 5 min, and electrophoresed on 4–20% SDS polyacrylamide gradient gel. After electrophoresis, the proteins were blotted onto a polyvinylidene difluoride membrane at 20 mA for 60 min using the Bio-Rad Mini-PROTEAN gel system. After blotting, the membrane was washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and blocked with TBS-T containing 5% skim milk for 1 h at room temperature. After washing with TBS-T, the membrane was probed with monoclonal anti-VEGFA20 antibody (1:200 diluted; Santa Cruz, United States) or monoclonal anti-GAPDH antibody (1:200 diluted; Santa Cruz) in TBS-T containing 3% skim milk at 4°C for 48 h with gentle shaking. Subsequently, after washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated polyclonal rabbit anti-mouse IgG (1:3,000 diluted; Zymed, CA) in TBS-T containing 3% skim milk at room temperature for 2 h. Finally, the membrane was washed with TBS-T and developed using an ImmunoStar® LD (FUJIFILM) Western blotting detection reagent (GE Healthcare, United Kingdom). The chemical luminescence of the membrane was detected using a C-DiGit™ system (Li-COR). Densitometric analysis was performed using Image Studio Digits ver. 4.0 (Li-COR). Relative protein expression was calculated by determining the ratio of each protein to GAPDH.

Measurement of Plasma Corticosterone and Vascular Endothelial Growth Factor Concentration

Plasma corticosterone and VEGF concentrations were measured using the corticosterone enzyme-linked immunosorbent assay kit (Cayman Chem., MI, United States) or the mouse VEGF ELISA Kit (Proteintech, United States) according to the manufacturer's instructions, respectively. In corticosterone ELISA, we measured each sample in duplicate using two plates, and the intra- and inter-assay coefficient of variations were 0.011 and 0.097, respectively. In VEGF ELISA, we measured each sample in duplicate using one plate, and the intra-coefficient of variations was 0.064.

Statistical Analyses

Statistical analyses were performed using Prism version 8 (GraphPad Software Inc., San Diego, CA, United States). Values are expressed as mean ± standard error of the mean (SEM). Comparisons of the two groups were performed using an unpaired two-tailed Student's *t*-test. Plasma corticosterone was analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test for *post hoc* analysis. Locomotor activity was analyzed using repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test for *post hoc* analysis. For experiment 4, a one-way ANOVA was used with Tukey's test corrected for multiple comparisons test. Statistical significance was assumed at p-values of < 0.05.

RESULTS

Comparison of the Physical Activity Amount in the Standard and Physical Inactivity Cages

We compared physical activity levels of mice that were housed in the standard and PI cages for 2 days *via* implanted nano-tag. **Figure 2A** shows the changes in physical activity levels per hour throughout 1 day. Physical activity per hour during the daytime was not significantly different in the two cages, whereas that of the nighttime was lower in the PI cages compared to the standard cage, and there were significant differences observed from 19 to 21 h (**Figure 2A**). Moreover, when the 1-day amount of physical activity was calculated separately into daytime and nighttime, that of the daytime was equal in two cages (**Figure 2B**), whereas that of the nighttime was significantly lower in the PI cage than in the standard cage ($p < 0.001$, **Figure 2B**).

Plasma Corticosterone Concentration During Housing in the Physical Inactivity Cage

We measured plasma corticosterone concentration to examine stress state in the mice housed in the PI cage long-term. The plasma corticosterone concentration did not alter before, 1 week after, or ten weeks after housing (**Figure 3B**).

Assessments of Cognitive Function and Depressive-Like State in Physical Inactivity Mice

For 10 weeks, we housed the mice in the PI cages to examine whether long-term housing in the PI cage affects cognitive function and depressive behaviors. Ten weeks of housing in the PI cage did not affect body weight ($p = 0.55$, **Figure 4B**) but significantly decreased food intake ($p = 0.04$, **Figure 4C**). Plasma corticosterone concentration measured at the housing end did not significantly differ between the two conditions ($p = 0.81$, **Figure 4D**). Interestingly, the PI mice showed a significant

reduction in the spontaneous alternation ratio in the Y-maze test ($p < 0.03$, **Figure 4E**) and immobility time in the CFCT ($p < 0.002$, **Figure 4F**) than the Cont mice. Furthermore, the SPT result showed that the Cont and PI mice had no significant difference in sucrose intake ($p = 0.46$); however, the PI mice had a significantly higher intake of water ($p < 0.001$). Therefore, the PI mice's preference ratio for sucrose was lower than that of the Cont mice ($p < 0.003$, **Figure 4G**). Incidentally, there was no difference in the immobility time in the FST between the two conditions ($p = 0.78$, **Figure 4H**). Details of the data discussed here are shown in **Supplementary Table 1**.

Effect of Regular Low-Intensity Exercise on Muscle Weight, Fat Mass Gain, Muscle Strength, and Endurance Running Capacity in Physically Inactive Mice

We examined the effect of regular low-intensity exercise on the cognitive function and depressive-like state of the mice housed in the PI cage. No significant difference in body weight, [$F_{(2, 24)} = 1.36$, $p = 0.28$, **Figure 5B**] at the time of dissection, and average food intake [$F_{(2, 24)} = 0.659$, $p = 0.53$, **Figure 5C**], during the housing period, was observed among the Cont, PI and PI + Ex mice. Plasma corticosterone concentrations at the time of dissection were not significantly different among the three groups [$F_{(2, 24)} = 0.962$, $p = 0.40$, **Figure 5D**]. Soleus muscle weight significantly decreased in the PI mice than in the Cont and PI + Ex mice [$F_{(2, 23)} = 6.411$, $p = 0.01$, **Figure 5E**], whereas no significant difference was noted between the Cont and PI + Ex mice. The extensor digitorum longus (EDL) muscle weight significantly decreased in the PI mice than in the Cont and PI + Ex mice [$F_{(2, 24)} = 8.142$, $p = 0.002$, **Figure 5F**], whereas no significant difference in gastrocnemius muscle weight was observed among the three groups [$F_{(2, 24)} = 1.880$, $p = 0.17$, **Figure 5G**]. In contrast, epididymal fat weight significantly increased in the PI mice than in the other two groups of mice [$F_{(2, 24)} = 5.334$, $p = 0.01$, **Figure 5H**]. Moreover, no significant differences in grip strength using all limbs were found among the three groups [$F_{(2, 24)} = 1.623$, $p = 0.22$, **Figure 6A**]. For grip strength using the forelimbs, the PI mice showed a significantly lower level than the PI + Ex mice [$F_{(2, 24)} = 3.789$, $p = 0.04$, **Figure 6B**]. The PI and PI + Ex mice showed a significantly shorter running time than the Cont group [$F_{(2, 24)} = 6.493$, $p = 0.006$, **Figure 6D**], whereas no significant difference was observed between the PI and PI + Ex mice. Details of the data mentioned here are shown in **Supplementary Table 2**.

Regular Low-Intensity Exercise Prevented Cognitive Decline and Depression-Like State *via* Physical Inactivity

We conducted four behavioral tests to examine the cognitive function and depressive-like state in mice. First, in the Y-maze test for examining working memory, the spontaneous alternation ratio was significantly lower in the PI mice than in the Cont and PI + Ex mice, and there was no difference between the Cont and PI + Ex mice [$F_{(2, 24)} = 8.301$, $p = 0.002$, **Figure 7A**]. Second, immobility time (%) in the CFCT for examination of long-term memory was significantly decreased in the PI mice than in the Cont mice [$F_{(2, 23)} = 3.351$, $p = 0.05$, **Figure 7B**]. Third, in the SPT to examine the depressive-like state in the mice, the SPT result showed that the Cont and PI mice had no significant difference in sucrose intake [$F_{(2, 24)} = 0.263$, $p = 0.77$]; however, the PI mice had a significantly higher intake of water [$F_{(2, 24)} = 4.660$, $p < 0.02$]. Therefore, the preference ratio for sucrose of the PI mice was lower than that of the Cont mice [$F_{(2, 24)} = 4.091$, $p < 0.03$, **Figure 7C**]. In the FST to examine depressive state, the Cont mice showed lower immobility time than the PI and PI + Ex mice, but the difference was not significant [$F_{(2, 24)} = 3.802$, $p = 0.04$, **Figure 7D**]. Lastly, in the EPM for examining anxiety, the time spent in the open arm significantly decreased in the PI mice than in the Cont mice [$F_{(2, 24)} = 3.320$, $p = 0.05$, **Figure 7E**]. However, the closed arm yielded no differences among the three groups [$F_{(2, 24)} = 0.211$, $p = 0.81$, **Figure 7F**]. Details of the data shown here are shown in **Supplementary Table 3**.

Regular Low-Intensity Exercise Prevented the Deterioration of Ki-67 Positive Cells and Surface Capillary Density by Physical Inactivity Housing

We measured the number of Ki-67 positive cells in the hippocampus to examine hippocampal neuronal cell proliferation. The number of Ki-67 positive cells in the PI + Ex mice was significantly higher than that in the PI mice, with no significant difference compared with the Cont mice [$F_{(2, 24)} = 6.965$, $p < 0.004$, **Figures 8A,B**]. Similarly, the number of Ki-67 positive cells in the PI mice was significantly lower than that in the Cont mice. Hippocampal surface capillary density, measured based on CD31-positive cells, also significantly decreased in the PI mice than in the Cont mice; the PI + Ex mice showed significantly higher levels of the PI mice [$F_{(2, 23)} = 17.68$, $p < 0.001$, **Figures 8C,D**].

Regular Low-Intensity Exercise Increased Plasma Vascular Endothelial Growth Factor Concentration and Hippocampal Brain-Derived Neurotrophic Factor and Vascular Endothelial Growth Factor mRNA Expression

Our immunohistochemical results showed that physical inactivity resulted in cognitive decline and a depressive-like state with decreased neuronal cell proliferation and angiogenesis in the hippocampus, which is consistent with a previous study result showing that hippocampal neurogenesis and angiogenesis are closely related (Heine et al., 2005). One of the contributing factors to hippocampal angiogenesis is VEGF; therefore, we examined changes in VEGF expression in the mice groups. Plasma VEGF concentration was significantly higher in the PI + Ex mice than

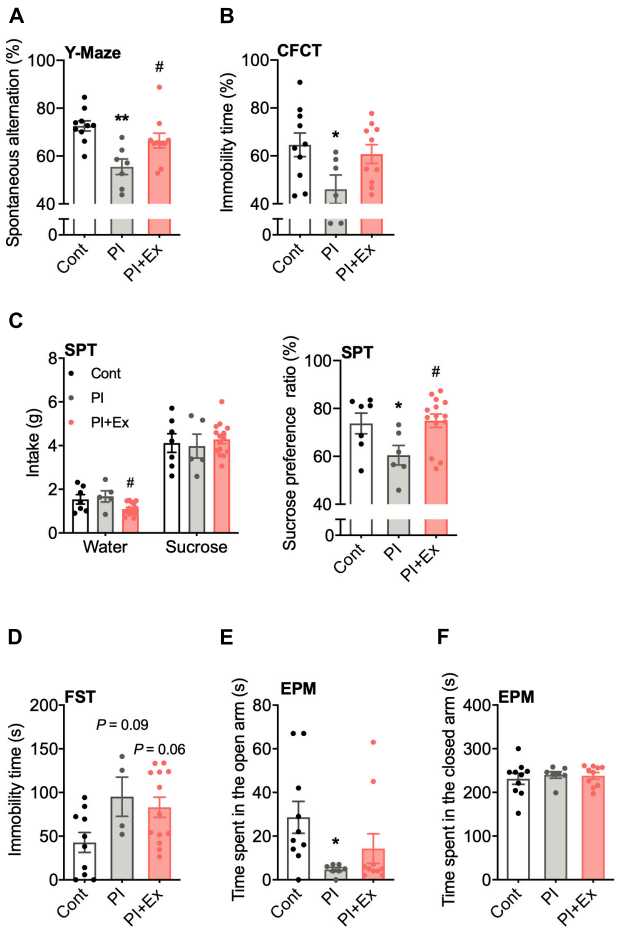

FIGURE 7 | Regular low-intensity exercise prevents cognitive decline and depressive state due to physical inactivity. **(A)** Accuracy rate in the Y-maze test. **(B)** Immobility time in the contextual fear conditioning test (CFCT). **(C)** Sucrose preference ratio in the sucrose preference test (SPT). **(D)** Immobility time in the forced swim test (FST). **(E,F)** Time spent in the open and closed arm in the elevated plus-maze test (EPM). All data are presented as the mean ± SEM (Cont mice, $n = 10$; PI mice, $n = 7$; PI + Ex mice, $n = 10$). Data were analyzed using one-way ANOVA with Tukey's *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$ in compared with the Cont mice and $^{\#}p < 0.05$ in comparison with the PI mice.

in the other two groups of mice [$F_{(2,\ 24)} = 6.897$, $p < 0.004$, **Figure 9A**]. VEGF protein levels in the gastrocnemius muscle did not differ among the three groups [$F_{(2,\ 24} = 0.7854$, $p = 0.47$, **Figure 9B**]. Hippocampal VEGF mRNA expression significantly decreased in the PI mice than in the Cont and PI + Ex mice [$F_{(2,\ 24)} = 10.54$, $p = 0.001$, **Figure 9C**]; no significant difference was evident between the Cont and PI + Ex mice. The same pattern was observed for hippocampal BDNF mRNA expression [$F_{(2,\ 24} = 6.745$, $p = 0.005$, **Figure 9D**].

DISCUSSION

One of the purposes of this study was to establish a valid experimental model to examine the effect of long-term physical inactivity on cognitive function and a depressive-like state. For this purpose, we made a special cage using acrylic plates, which we named as the physical inactivity (PI) cage. Housing the mice in the PI cage decreased the space in which the mice could freely move, and measurements of the mice's locomotor activity using a nano-tag showed that mice that were housed in the PI cage decreased nighttime physical activity to about 50% of those housed in a standard cage (**Figure 2B**). In addition, to measure physical activity, the mice were abdominally implanted with a nano-tag. Although a nano-tag is only about 3 g, it is about 1/10 of the mouse's body weight. Therefore, the implantation of the nano-tag weighing 3 g could have restricted physical movement and lowered the mice's physical activity; consequently, the mice's amount of physical activity while housed in the standard cage could have been somewhat lower than that of the mice not implanted with a nano-tag. Thus, the decrease in physical activity by housing in PI cage could have been more than 50%. Therefore, these results showed that the size of the space in which the mice are daily residents is a limiting factor for the physical activity of the mice, and we think that housing the mice in the PI cage is a valuable way to cause physical inactivity in mice.

In the present study, the plasma corticosterone concentration of the mice housed in the PI cage did not significantly change during 10 weeks, from the start of housing to its end (**Figure 3B**). Moreover, no increase in plasma corticosterone concentration was observed even in the other two experiments (**Figures 4D**, **5D**). Plasma corticosterone concentration is a primary indicator of stress state; therefore, this result suggests that housing in the PI cage would not cause severe stress to the mice. The reason that isolation stress during housing in the PI cage was alleviated is speculated as follows. First, the mice could visually confirm other mice next to them through a transparent acrylic wall (**Figure 1**). Second, they could touch their noses through holes drilled in the wall (**Figure 1**). It is important to note that this study did not measure stress indications other than plasma corticosterone concentration, such as plasma ACTH concentration (Lightman et al., 2020) and immunohistochemical analysis of Iba-1-positive cells in the hippocampal dentate gyrus (Rivera et al., 2020). Therefore, we need to clarify the existence and absence of the stress of the mice housed in the PI cage by measuring other stress indicators in future studies.

Long-term housing in the PI cage caused loss of muscle weight (soleus and EDL muscles) and epididymal fat mass gain (**Figures 5E,F,H**), whereas body weight and gastrocnemius muscle weight were not altered (**Figures 5B,G**). Moreover, it resulted in a reduction in muscle strength (not significant) (**Figures 6A,B**) and endurance exercise capacity (**Figure 6D**). In contrast previous studies reported that hindlimb suspension (Gaignier et al., 2014; Marzuca-Nassr et al., 2019) or cast fixation (Lang et al., 2012; Morimoto et al., 2013; Ye et al., 2013), even within 1–2 weeks, resulted in increased plasma corticosterone levels (Steffen and Musacchia, 1987) and atrophy of all muscles. Judging from these results, hindlimb or cast fixation results in quick disuse muscle atrophy with severe stress in a short-term period, whereas physical inactivity due to housing in the PI cage long-term would result in mild disuse muscle atrophy, energy consumption reduction, and functional deterioration of

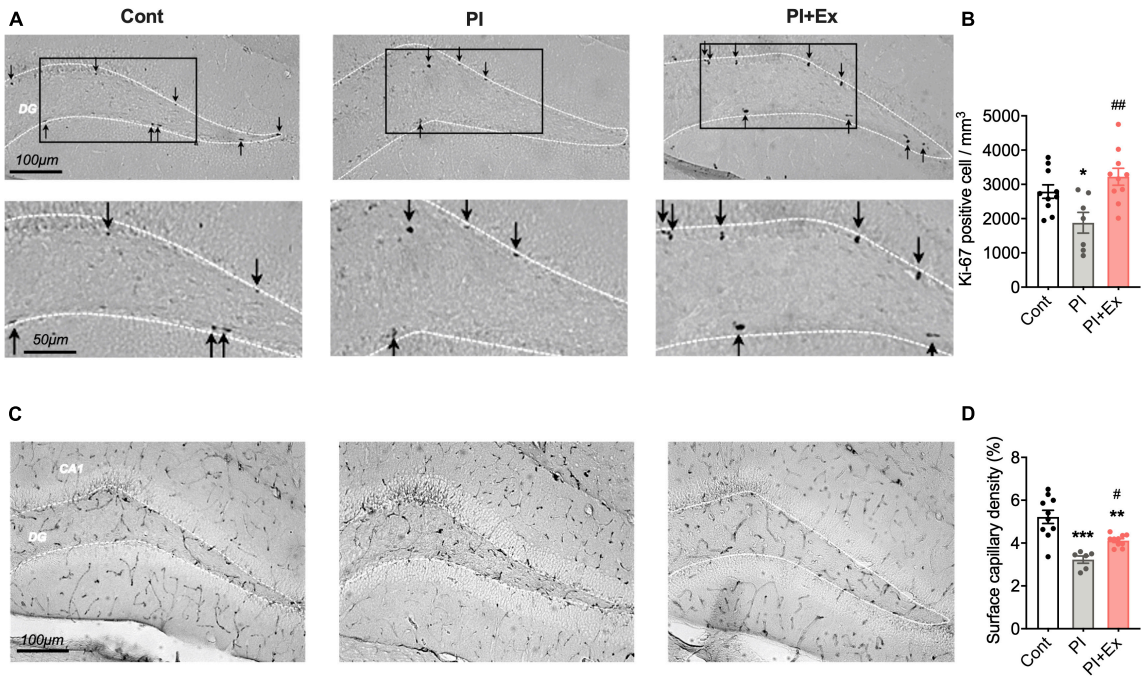

FIGURE 8 | Regular low-intensity exercise prevents the decrease in Ki-67 positive cells and surface capillary density in the hippocampus due to physical inactivity housing. **(A,B)** Ki-67 positive cells in the hippocampus dentate gyrus. **(C,D)** Surface capillary density measured by CD31-positive cells in the hippocampus. All data are presented as the mean ± SEM (Cont mice, $n = 10$; PI mice, $n = 7$; PI + Ex mice, $n = 10$). Data were analyzed using one-way ANOVA with Tukey's *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with the Cont mice and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ in comparison with the PI mice.

muscles. Furthermore, the former models provide severe stress to animals; therefore, these are considered inappropriate for examining the independent influence of physical inactivity on cognition decline and depression. Additionally, our experimental model would be a valuable strategy for investigating physical inactivity's physiological and neurological influence on biogenic function, but we examined only muscle weight in the present study and did not study other indicators such as cross-sectional muscle area or muscle fiber composition. Therefore, we need to elucidate whether housing in the PI cage would result in muscle atrophy by measuring indicators other than muscle weight.

Previous studies have suggested that hippocampal neuronal proliferation decreases are closely related to cognitive decline *via* aging (van Praag et al., 2005; Horowitz et al., 2020) or chronic stress (Nakajima et al., 2009; Muto et al., 2014; Lee et al., 2018). Furthermore, chronic stress causes a decrease in hippocampal blood vessel density, which leads to a depressive-like state (Kiuchi et al., 2012). In the present study, long-term physical inactivity also caused cognitive decline and a depressive-like state, and the hippocampus's decreased neurogenesis and vascular density were simultaneously observed. Therefore, we expected that the cause of this behavioral deterioration would be due to decreased neurogenesis and decreased vascular density in the hippocampus. The following two points were expected as the factors that triggered these hippocampal changes. The first is psychological stress induced by limiting the amount of physical activity. There is a possibility that psychological stress could result from the mice being unable to move as desired. The second was a reduction in the amount of myokines released from skeletal muscles due to the decreased use of these muscles from physical inactivity. Myokines, especially irisin, are re-released from skeletal muscles, and their release is increased by exercise (Jedrychowski et al., 2015) and contributes to improving cognitive function (Lourenco et al., 2019). The decreased use of skeletal muscle due to housing in the PI cages might have suppressed myokines released from skeletal muscles, but this study has not verified this point. The purpose of this study was to uncover the direct influences of physical inactivity on cognitive function and depressive-like state; however, at this moment, we can only show results including the influence of some additional stress.

Regular low-intensity exercise prevents the suppression of neuronal cell proliferation and angiogenesis in the hippocampus due to long-term physical inactivity, preventing cognitive decline and depressive behaviors. Newly generated neuronal cells are observed mainly near the capillaries in the hippocampus (Heine et al., 2005), and hippocampal neurogenesis and angiogenesis are closely related to each other (Heine et al., 2005). Furthermore, a previous study showed that regular, moderate exercise prevented neuronal cell proliferation and angiogenesis in the hippocampus (Kiuchi et al., 2012). Based on these results, we hypothesized that regular low-intensity exercise could prevent cognitive decline and the onset of a depressive-like state by inhibiting the decrease in neuronal cell proliferation and angiogenesis due to long-term physical inactivity.

In this study, plasma VEGF concentration and hippocampal VEGF mRNA expression were increased in the PI + Ex mice

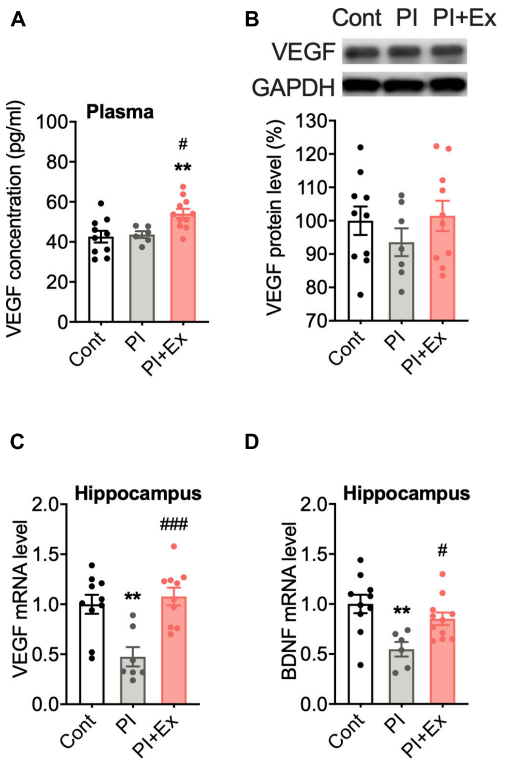

FIGURE 9 | Regular low-intensity exercise increased plasma VEGF concentration and hippocampal BDNF and VEGF mRNA expression. **(A)** Plasma VEGF concentration. **(B)** Representative gel image of western blot analysis for VEGF protein expression in the gastrocnemius muscle. **(C)** Relative mRNA level for VEGF in the hippocampus. **(D)** Relative mRNA level for BDNF in the hippocampus. All data are presented as the mean ± SEM (Cont mice, $n = 10$; PI mice, $n = 7$; PI + Ex mice, $n = 10$). Data were analyzed using one-way ANOVA with Tukey's *post-hoc* test. $^{**}p < 0.01$, in comparison with the Control mice and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ in comparison with the PI mice.

(**Figures 7A,C**). In a previous study, VEGF-overexpressing transgenic mice showed enhanced hippocampal neurogenesis (Udo et al., 2008), and intraventricular VEGF administration could also enhance hippocampal neurogenesis levels (Sun et al., 2003, 2006; Licht et al., 2011). Conversely, both hippocampus-specific VEGF knockdown (Sun et al., 2006) and VEGF receptor antagonist-administered mice (Jin et al., 2002) showed decreased hippocampal neurogenesis. Our previous study also showed that administration of the VEGF receptor antagonist SU1498 inhibits the increase in hippocampal cell proliferation resulting from regular exercise training (Kiuchi et al., 2012). The origin of plasma VEGF is likely the skeletal muscle and liver. The VEGF in skeletal muscle did not differ among the three groups in our study (**Figure 7B**). Moreover, regardless of the intensity, hepatic blood flow would decrease (Dyke et al., 1998). We expected that the decreased hepatic blood flow would decrease liver oxygen levels, followed by hypoxia-induced factor-1 and VEGF expression. Nevertheless, we could not examine the hepatic VEGF content as we did not collect liver samples.

A decrease in hippocampal BDNF results in cognitive decline and depressive-like state, whereas hippocampal BDNF recovery through several methods, including regular exercise, drug administration, and transgenic modification, restores cognitive function. In this study, the decreased hippocampal BDNF mRNA expression due to physical inactivity was restored by regular exercise (**Figure 7D**). This result indicates that physical inactivity causes cognitive decline and a depressive-like state by decreasing neuronal cell proliferation and BDNF expression in the hippocampus. In contrast, regular exercise prevents decreased hippocampal BDNF expression and thus maintains hippocampal cell proliferation and cognitive function.

Limitations

The purpose of this study was to explore the direct influences of physical inactivity on cognitive function and a depressive-like state; however, at this moment, we can only show results including the influence of some external stress. In animal experiments, it is difficult to investigate the effect of physical inactivity on cognitive function and a depressive-like state without causing the experimental animals stress, especially isolation stress. To achieve the purpose, we will need further to alleviate the influence of isolation stress in the future. For this purpose, based on the result of the diurnal rhythm of the physical activity obtained, the cage size did not affect the daytime physical activity; therefore, to exclude isolation stress, it may be valid to rear mice as a group in a standard cage during the day and separately in the PI cages during nighttime. Furthermore, to distinguish the effect of the isolation stress and physical inactivity, it will need to compare the stress markers and behavioral changes between the mice housed in the PI cage and the mice housed separately. It also will be necessary to compare the effect of cage size on the changes in stress markers and behaviors. In addition, we also need to examine stress markers other than plasma corticosterone. Moreover, we suppose that the primary factor in inducing cognitive decline and onset of a depressive-like state due to physical inactivity is likely the reduction of myokines released from skeletal muscles resulting from skeletal muscle disuse. An experiment using neutralizing antibodies for irisin will be necessary to test this hypothesis. The present study is the first step in investigating the direct influences of physical inactivity on cognitive function and depressive-like state.

CONCLUSION

To investigate the effect of physical inactivity on cognitive function and depressive-like state, we housed mice in the PI cage. Daily physical activity was decreased in the PI mice to about 50% of the control mice. Housing in the PI cage long-term resulted in cognitive decline and a depressive-like state with reduced hippocampal neuronal cell proliferation, hippocampal blood density, plasma VEGF level, hippocampal VEGF, and BDNF mRNA expression. Regularly low-intensity exercise restored the decreased hippocampal factors, preventing cognitive decline and a depressive-like state. The physical inactivity model *via* housing in the PI cage showed in the present study may become an

adequate and valuable experimental model for investigating the effect of physical inactivity on brain function, particularly cognitive function.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Nippon Medical School (approval no. 28-023).

AUTHOR CONTRIBUTIONS

TM and JK designed the study and wrote the manuscript. TM, JK, and JP performed the research and analyzed the data. All authors contributed to the article and approved the submitted version.

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Trauma Disrupts Reinforcement Learning in Rats—A Novel Animal Model of Chronic Stress Exposure

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Trauma, as well as chronic stress that characterizes a modern fast-paced lifestyle, contributes to numerous psychopathologies and psychological problems. Psychiatric patients with traumas, as well as healthy individuals who experienced traumas in the past, are often characterized by diminished cognitive abilities. In our protocol, we used an animal model to explore the influence of chronic trauma on cognitive abilities and behavior in the group of 20 rats (Rattus norvegicus). The experimental group was introduced to chronic (12 consecutive days) exposure to predator odor (bobcat urine). We measured the reinforcement learning of each individual before and after the exposition *via* the Probabilistic Selection Task (PST) and we used Social Interaction Test (SIT) to assess the behavioral changes of each individual before and after the trauma. In the experimental group, there was a significant decrease in reinforcement learning after exposure to a single trauma (Wilcoxon Test, $p = 0.034$) as well as after 11 days of chronic trauma (Wilcoxon-test, $p = 0.01$) in comparison to pre-trauma performance. The control group, which was not exposed to predator odor but underwent the same testing protocol, did not present significant deterioration in reinforcement learning. In cross-group comparisons, there was no difference between the experimental and control group in PST before odor protocol (U Mann-Whitney two-sided, $p = 0.909$). After exposure to chronic trauma, the experimental group deteriorated in PST performance compared to control (U Mann-Whitney Two-sided, $p = 0.0005$). In SIT, the experimental group spent less time in an Interaction Zone with an unfamiliar rat after trauma protocol (Wilcoxon two-sided test, $p = 0.019$). Major strengths of our models are: (1) protocol allows investigating reinforcement learning before and after exposition to chronic trauma, with the same group of rats, (2) translational scope, as the PST is displayed on touchscreen, similarly to human studies, (3) protocol delivers chronic trauma that impairs reward learning, but behaviorally does not induce full-blown anhedonia, thus rats performed voluntarily throughout all the procedures.

Keywords: reinforcement learning, trauma, PTSD, predator odor, chronic stress

INTRODUCTION

Throughout life, the environment puts numerous stressors on every living organism. In humans, extreme stress (trauma) captures a range of severe adverse experiences, such as physical, sexual, or emotional abuse, neglect, parental death, bullying, or omission by caregiver during childhood. Trauma contributes to the development of numerous mental disorders such as posttraumatic stress disorder (PTSD), anxiety disorders, schizophrenia, personality disorders, mood disorders (Jansen et al., 2016; Misiak et al., 2017). It is estimated that prevalence of PTSD reaches 7% in general population (McLaughlin et al., 2015), while in subgroups exposed to severe psychological trauma numbers are even more prominent, for example, 10% of US veterans meet criteria of PTSD (Mota et al., 2016) as well as 60% minor refugees in Germany that sought general medical treatment (Veeser et al., 2021). In the general population, only a small proportion of individuals with a positive history of traumatic events develop full-blown PTSD (Breslau, 2009). Trauma affects cognitive abilities (Petkus et al., 2018; Aas et al., 2019), disrupts the immune system (Mehta et al., 2020), causes structural changes in the brain (Assogna et al., 2020), affects the severity of symptoms among those with mental disorders (Duhig et al., 2015; Ay and Erbay, 2018; Bailey et al., 2018). Chronic stress, defined as an exposition to a series of stressful or potentially traumatic events, characterizes a modern, fast-paced western lifestyle (Matosin et al., 2017). Chronic stress turns out to be closely related to numerous health issues: obesity, diabetes, mental disorders, psychological deficits, substance dependence (Sinha, 2008; Farag and Gaballa, 2011; Misiak et al., 2017; Bielawski et al., 2019). All are major epidemiological health concerns that generate enormous public cost (Simon et al., 2006; Farag and Gaballa, 2011; Laramée et al., 2013; Masodkar et al., 2016). The purpose of this study is to present a novel protocol to examine cognitive impairment in reinforcement learning as chronic trauma progresses. We use a simplified Probabilistic Selection Task (PST) to approximate our model to human studies. In humans, experimental studies of PTSD, chronic stress, and trauma are limited. Therefore, our research is to explore the translational scope of PTSD studies in rodents. We want to test whether rats will perform voluntarily while exposed to chronic trauma. If so, our aim is to study rats' ability to learn the PST protocol, as well as their ability to adapt to a system, where interaction with a touchscreen is related to reward collection. Our procedure examines reward learning before and after exposure to chronic trauma, with the same group of rats. This approach allows us to measure cognitive disruptions as the trauma progresses. We hypothesize that rats exposed to trauma will perform poorer in PST, in comparison to their performance before exposure to chronic trauma. Moreover, we want to explore whether a single exposure to trauma will affect cognitive functioning. Furthermore, we hypothesize that traumatized individuals will be less socially oriented during Social Interaction Test (SIT), compared to the control.

MATERIALS AND METHODS

Theoretical Background

Trauma, Cognition, and Chronic Stress Rationale

Medically oriented understanding of psychological trauma is strictly related with PTSD diagnosis (Yehuda, 1998), while in psychoanalytic approach trauma is a powerful stimulus, that breaches one's psychological defense mechanisms, and induces experience of helplessness (Rothgeb, 1971). In both definitions trauma is an extreme stress, that is beyond one's ability to cope with. An abundant literature presents negative impact of trauma on cognitive functions in patients with psychosis (Lysaker et al., 2001; Schenkel et al., 2005; Shannon et al., 2011) and among healthy individuals who experienced trauma in the past (Majer et al., 2010; Vasilevski and Tucker, 2016; Petkus et al., 2018). Trauma and prolonged (chronic) stress activate the hypothalamic-pituitary-adrenal (HPA) axis *via* the rise of corticosteroids, activate the endocannabinoid system, and indirectly affect dopamine bursts in the striatum and medial prefrontal cortex (Joëls et al., 2012; Bielawski et al., 2019). Different regions of the brain (for example hippocampus, amygdala, medial prefrontal cortex, hypothalamus) involved in stimulus recognition, memory, and learning are affected by increased detrimental corticosteroids rise during chronic or acute stress (Pruessner et al., 2017; Bielawski et al., 2019). The neurobiology of trauma and its impact on cognitive abilities is complex, and studies in human subjects have certain limitations. Thus, several animal models have been developed to assess symptoms associated with exposure to trauma and the development of PTSD (Whitaker et al., 2014; Harro, 2018; Planchez et al., 2019). The Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-V) delivered by the American Psychiatric Association (APA) presents four clusters of symptoms of PTSD: intrusive recollection of the original traumatic event, avoidance of trauma-related reminders, negative changes in cognition and mood, and alterations in arousal or reactivity, each of which must start or be significantly exacerbated after exposure to the traumatic event (Roehr, 2013). The variety of animal models put its focus on different aspects of PTSD symptomatology, such as contextual avoidance (Albrechet-Souza et al., 2020), changes in arousal and reactivity (Knox et al., 2012), and behavior alterations (Krishnan et al., 2007). These models measure different parameters after the exposition to stress. Our approach is to measure cognitive and behavioral parameters as chronic trauma progresses. That way, an animal model gives us an opportunity to expose rats to chronic stress, as we measure their cognitive functions simultaneously. Chronic stress lacks a clear definition, but most authors agree that it is an exposition to a series of intense, potentially traumatic experiences or involvement in prolonged stress situations that leads to psychopathologies and/or adverse medical conditions (Matosin et al., 2017). Chronic stress is widely used in animal models of anxiety disorders, depression, and PTSD (Saavedra-Rodríguez and Feig, 2013; Reber et al., 2016; Wang et al., 2021). In humans, prolonged stress is an important factor in etiopathology of different mental disorders (Matosin et al., 2017; McEwen, 2017;

Ross et al., 2017), for example chronic stress can induce mild PTSD symptoms in humans (Davidson and Baum, 1986). Stress influences the ability to learn from rewards among those with a familial predisposition to psychosis and individuals with major depressive disorder (Reinen et al., 2021). Furthermore, chronic stress induces hyper inflammation, thus being discussed to enhance susceptibility to infectious diseases such as COVID-19 (Lamontagne et al., 2021), or mental diseases linked to immune system dysregulations (Dennison et al., 2012). Chronic exposure to trauma is particularly harmful; many individuals repeatedly exposed to traumatic events carry a heavy burden of psychopathologies (Sharhabani-Arzy et al., 2003; Éthier et al., 2004; Salcioglu et al., 2017). In our experiment, we expose male Wistar Rats to chronic trauma for 12 consecutive days. In the literature, there are animal models of PTSD that reveal alteration in cognitive performance, although they often apply a single prolonged stress procedure (George et al., 2015). Indeed, single exposure to predator odor is sufficient to induce trauma (Albrechet-Souza and Gilpin, 2019), but our goal is to mimic chronic stress, thus our protocol's prolonged exposure to stressful stimulus with parallel cognitive examination.

Probabilistic Selection Task and Social Interaction Test

In humans, the Probabilistic Selection Task (PST) was shown to be associated with dopaminergic effects on learning (Frank et al., 2007). Positron emission tomography and functional magnetic resonance imaging studies showed that reinforcement-based decisions are associated with signaling in the striatum and prefrontal cortex (Jocham et al., 2011; Kasanova et al., 2018). Furthermore, PST was used to assess learning deficits among those with PTSD (Myers et al., 2013). During PST, participants are presented stimulus pairs and learn to choose one of them. After each choice, probabilistic feedback follows the choice to

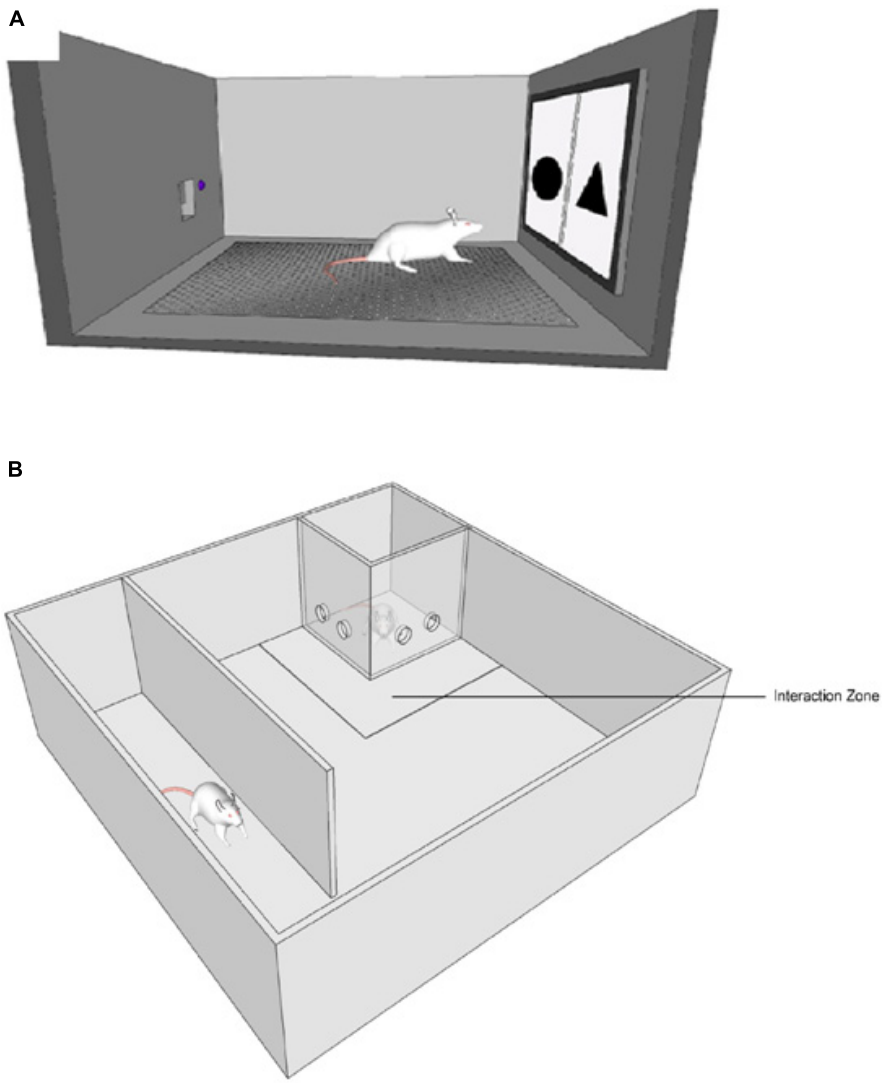

FIGURE 1 | (A) Probabilistic Selection Task testing chamber. **(B)** Social Interaction Test testing chamber.

indicate whether it was correct or incorrect. PST (and its different variants) are widely used in animal studies—in rodents stimulus selection is most often recorded *via* nose poke in aperture (Amitai et al., 2014) or by pressing the lever (George et al., 2015; Seib et al., 2020), while in humans selection is usually done *via* tap on a touchscreen or pressing a button on a keyboard (Frank et al., 2004).

The Social Interaction Test (SIT) is a popular method to assess levels of anxiety, social interaction, locomotor activity, and arousal in rodents (File and Seth, 2003). In our experiment, an examined rat is introduced into the test box with a tunnel, open field arena, and Interaction Zone with unfamiliar rat. Examined rat behavior is monitored; time spent in different parts of the test arena, number of droppings, or freezing behavior. Our model explores cognitive changes among Wistar Rats through the PST, as well as anxiety level and social interaction through the Social Interaction Test. We used SIT procedure similar to the one in social defeat experiments (Golden et al., 2011; Toyoda, 2017).

Predator Odor as Traumatizing Factor

In our study, we use an animal model with predator odor exposure that produces behavioral, physiological, and molecular alterations that recapitulate many of the same alterations observed in PTSD patients (Cohen et al., 2012). We use bobcat urine as a stressor, it is a well-established model used in a series of studies done by Gilpin and colleagues (Albrechet-Souza and Gilpin, 2019). Bobcat urine contains the biogenic amine 2-phenylethylamine, which activates specific receptors within the rodent olfactory cortex, the trace amine-associated receptor 4 (TAART4), and can induce avoidance behavior in rats and mice (Ferrero et al., 2011). Furthermore, bobcat urine activates the amygdala-piriform transition area, which is responsible for increases in circulating stress hormones (Kondoh et al., 2016). In 1993, Yehuda and Antelman developed 5 criteria that animal models must meet, to parallel PTSD-related phenotypes: (1) Even a brief stressor should be able to induce biological and behavioral sequelae of PTSD, (2) The stressor should be able to produce PTSD-like sequelae in a dose-dependent manner, (3) Stressors should produce biological alterations that persist over time or become more pronounced with passage of time, (4) The stressor should induce biobehavioral alterations that have the potential for bidirectional expression, (5) Interindividual variability in response to a stressor should be present either as a function of experience, genetics, or an interaction of the two (Yehuda and Antelman, 1993). Studies done with bobcat urine meet most of those criteria (Albrechet-Souza and Gilpin, 2019), and are well discussed in the context of animal PTSD model (Albrechet-Souza et al., 2020, 2021). Taking the literature mentioned above, we feel confident using this type of traumatizing stimulus in our protocol.

Subjects

In our procedure, we used male Wistar Rats (Animal Research Center, Wrocław Medical University, PL) in a total number of 26 individuals ($n = 26$), although 20 individuals were included in our experiment ($n = 20$). Rats arrived at the age of 39–42 days, weighing 210–245 g at the day of arrival, were submitted to a handling period (7 days), and then entered P0. Six individuals did not meet the criteria to enter the P1, and were excluded during P0. Excluded animals either: (1) did not learn the tapping procedure throughout phase 0 or (2) presented freezing behavior during 3 consecutive days. Due to housing conditions and experimental procedure, the exclusion of a rat resulted in the exclusion of its cotenant. Therefore, even though $n = 3$ rats met the exclusion criteria, the total sum of $n = 6$ individuals was excluded.

A random group of rats ($n = 10$) participated as a control group, the second group ($n = 10$) participated as an experimental group ($n = 10$). Rats were pair housed on a non-reversed 12 h/12 h light/dark cycle (lights off at 7 p.m.). All behavioral tests were constructed during the light period. Rats had *ad libitum* access to food (dry pellets) and water.

The experiment was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Local Ethics Committee for Animal Experiments, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

Testing Chambers

The PST chamber was part of the device built by our team to measure PST in rats. It had a perforated metal floor that allowed animals to move freely and comfortably. Under the perforated floor there was a compartment where a sponge with odor could have been placed. The walls and floor of the chamber were easy to sanitize and safe for the animals to explore. The front wall had a hole, where a touchscreen apparatus displayed stimuli. Opposite the front wall, there was a feeder and a diode. Feeder was the place where rewards was delivered, a diode signaled when reward was about to be delivered (see **Figure 1A**).

SIT chamber was constructed from polyvinyl chloride (PCV) and Plexiglas. The main structure was a square 90 × 90 × 40 cm (length × width × height). Inside, there was a PCV wall 70 × 30 cm (length × height) that formed a tunnel. Furthermore, two additional transparent Plexiglas walls (20 × 30 cm) formed a closed space in one of the corners, where a new and unfamiliar rat was trapped (see **Figure 1B**). The 25 cm from plexiglas walls was marked as an "interaction Zone."

Procedure

PST- one pair of stimuli is presented in random order arrangement (left of right side of the screen) (see **Figure 2B**). Rats learned to choose one pair. Feedback was probabilistic; it means that in BC trials, a choice of stimulus B results in 90% positive feedback (10% negative feedback), while choice of stimulus C results in 90% negative feedback (10% positive feedback). Feedback follows the choice to indicate if it was correct (reward) or incorrect (punishment). The correct choice resulted in reward—a drop of sweet protein shake (Strawberry Nutridrink Protein, NUTRICIA, Poland). Incorrect choice resulted in punishment—lack of reward. The touchscreen was 26.5 cm width × 17 cm height and "tappable"—nose poke, strike, or touch with paw resulted with stimulus selection. When the stimulus was selected, the touchscreen went black for 8 s and a reward was delivered to the feeder, simultaneously with a light signal.

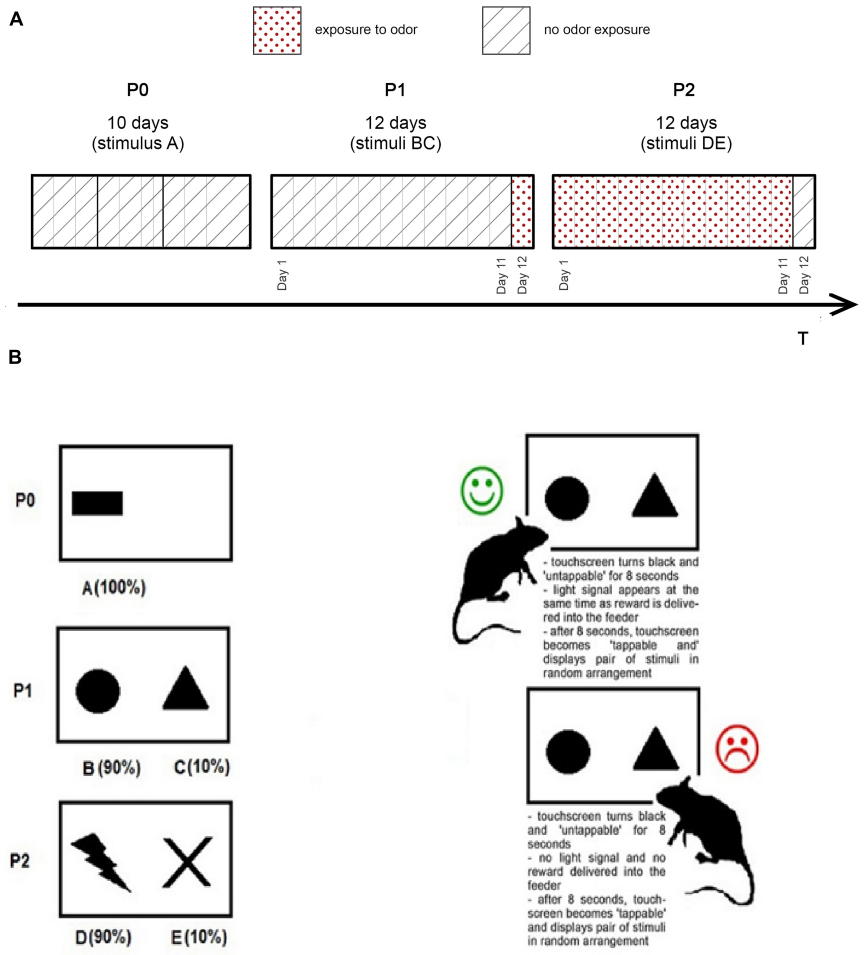

FIGURE 2 | (A) Experiment time schedule. **(B)** Probabilistic Selection Task procedure. P0, Phase 0; P1, Phase 1; P2, Phase 2.

After 8 s, the touchscreen displayed a randomly arranged pair of stimuli again.

SIT was performed twice throughout the experiment, P1 Day 6 and P2 Day 6. Examined individual was placed at the beginning of the tunnel. The session lasted 10 min and was videotaped.

Experimental Design

The rats were subjected to 1 week of handling before the phase 0. During handling sessions, rats were exposed to a sweet liquid, to adapt with sweet reward and feeder mouthpiece. An experiment consisted of 3 phases: phase 0 (P0), phase 1 (P1), and phase 2 (P2) (see **Figure 2A**). Each rat was examined *via* PST in the testing chamber once every day. Our protocol is a variation of the autoshaping task described by Horner et al. (2013).

Phase 0

P0 lasted 10 days and was designed to teach each animal the experimental procedure. During Day 1–3, the paired rats (according to the pair housing) were placed in the testing chamber to accommodate. The rats were able to explore the chamber for 20 min and collect rewards. During the first 6 days, the touchscreen displayed one visual stimulus on the left or right side (see **Figure 2B**). During the first 6 days, the rest of the touchscreen was "untappable"—there was no selection when tap occurred outside the stimulus sector. From 4 to Day 10, the rats were placed in the testing chamber separately, 10 min each.

Throughout Day 7–10 the stimulus was randomly displayed on the left or right side of the touchscreen, although the whole surface of the touchscreen was tappable. Tap delivered within the sector outside of the stimulus resulted in punishment—touchscreen went black for 8 s, no reward was delivered into the feeder. After 8 s, the touchscreen displayed the stimulus again randomly (left or right).

Phase 1

P1 lasted 12 days. Throughout P1, a pair of stimuli (B and C) was used in PST (see **Figure 2B**). Each animal was placed in the testing chamber for 20 min or until the session was completed. After each session, the testing chamber was thoroughly cleaned with disinfectant. The last day (P1 day 12) animals were exposed to predator odor, a sponge soaked with 3 ml of bobcat urine (Lynx rufus; Maine Outdoor Solutions, Hermon, ME, United States)

was placed on the testing chamber floor. In the control group, sponges were not soaked with bobcat urine.

Phase 2

P2 lasted 12 days. Throughout P2, a pair of stimuli (D and E) was used in PST (see **Figure 2B**). Each animal was placed in the testing chamber for 20 min or until the session was completed. After each session, the testing chamber was thoroughly cleaned with disinfectant. Throughout P2, a sponge soaked with bobcat urine (Lynx rufus; Maine Outdoor Solutions, Hermon, ME, United States) was placed under the testing chamber floor. The Last day (P2 Day 12) the animals were not exposed to predator odor. Control rats are treated identically to rats exposed to odors, but the sponges were not soaked with bobcat urine.

Data Collection

During the experiment, the rats performed PST once a day. Each session had 20 trials, the sessions ended when the last trial was completed or when 20 min passed. P1 and P2 lasted 12 days; we measured performance of each rat during 1, 11, and Day 12 (see **Figure 2A**). During those days, we recorded the number of wins (rewards delivered) and loses (punishment received).

In the experimental group, Day 1 was the day when a novel pair of stimuli was presented for the first time. Day 12 was the last day with a pair of known stimuli, but with changed environmental factors (odor or no odor exposure). Thus, P1 Day 1 was the first day when stimuli BC were displayed during PST, without exposure to odor. Day 11 of P1 was the day when stimuli BC were displayed without odor for the last time. P1 Day 12 was the day when BC stimuli were displayed for the last time, but this time with odor exposure. Accordingly, Day 1 ofP2 was the first day when stimuli DE were displayed during PST sessions, with odor exposure. P2 Day 11 was the day when DE stimuli were displayed with odor for the last time. P2 Day 12 was the day when stimuli DE were displayed for the last time, but this time without exposure to odor (see **Figure 2A**).

Behavioral Analysis

Video records were scored by the independent observer, who used stopwatch to measure the time spent in the Interaction Zone of each rat. Interaction Zone was outlined on the SIT floor. Crossing the line with hind limbs was considered as entry into the Interaction Zone.

Statistical Analysis

Analysis and interpretation of behavioral data acquired *via* PST is commonly aided by different variants of theoretical Q learning models (Frank et al., 2004; Frank, 2006; Frank and Claus, 2006; Brown et al., 2018; Kane et al., 2019; Metha et al., 2020). In this way, the research hypothesis is expressed as a set of mathematical equations that govern the analysis of the data. However, the theoretical model introduces its own assumptions and requires advanced routines to adjust the model to the dataset, which may bias the results in an unpredictable manner. Since our study involves a small amount of data, we decided to rely only on directly measurable variables, making the analysis model independent; thus, we present our data without a computational framework.

The test score of each individual was calculated during Days 1, 11, and 12—ratio of the gained rewards to all trials taken that day

$$T\ score_{Dx} = \frac{N\ rewards_{Dx}}{N\ rewards_{Dx} + N\ losses_{Dx}},$$

where $N\ rewards_{Dx}$ is the total number of rewards received during day X (Dx) and $N\ losses_{Dx}$ is the total number of punishment received during day X (Dx).

Then, we calculated the WinRatio of each individual for P1 and P2. WinRatio was a difference between Test score Day 11 and Test Score Day 1:

$$WinRatio = \left[\frac{N\ rewards_{D11}}{N\ rewards_{D11} + N\ losses_{D11}}\right] - \left[\frac{N\ rewards_{D1}}{N\ rewards_{D1} + N\ losses_{D1}}\right]$$

Day 1 and Day 11 test scores (used to calculate individual WinRatios) are presented in **Figure 3A**. Each rat's P1 WinRatio and P2 WinRatio is presented numerically in **Figure 3B**. Days 11 and 12 test scores are presented in **Figure 4**.

Due to a low number of rats and possibly non-normal distribution of variables, we used non-parametric statistical tests. To compare the performance of PST during P1 and P2 of the same rat, we used the Wilcoxon two-sided test. In cross-group comparisons, the U-Mann-Whitney two-sided test was used. Behavioral results were analyzed using the Wilcoxon two-sided test to compare times each rat spent in an Interaction Zone before and after the trauma, U-Mann-Whitney two-sided test was applied for cross-group comparisons. The statistical significance level was established at $p < 0.05$.

Statistical analysis was performed using the scipy.stats library belonging to the Python programming language ecosystem.[1]

RESULTS

With each individual's WinRatio for P1 (no odor) and P2 (with odor), we compared reinforcement learning before (P1) and after (P2) exposure to trauma in the experimental group, as well as reinforcement learning in the control group (see **Figure 3B**). In the experimental group, WinRatio during P1 was significantly greater than during P2 (Wilcoxon test, $p = 0.01$). In the control group, there was no significant difference in WinRatio between P1 and P2 (Wilcoxon Two-Sided Test, $p = 0.73$). In cross-group comparisons, the control group had a higher P2 WinRatio than experimental group P2 WinRatio (U Mann-Whitney Two-sided, $p = 0.0005$). There was no significant difference between the experimental P1 WinRatio and the control P1 WinRatio (two-sided Mann-Whitney U, $p = 0.909$). In general, both groups WinRatios are presented in **Figure 5A**.

The test score was calculated for Day 12 in P1 and P2 (see **Figure 4**). In the experimental group, the P1 Day 12 Test score

[1]https://docs.scipy.org/doc/scipy/reference/stats.html

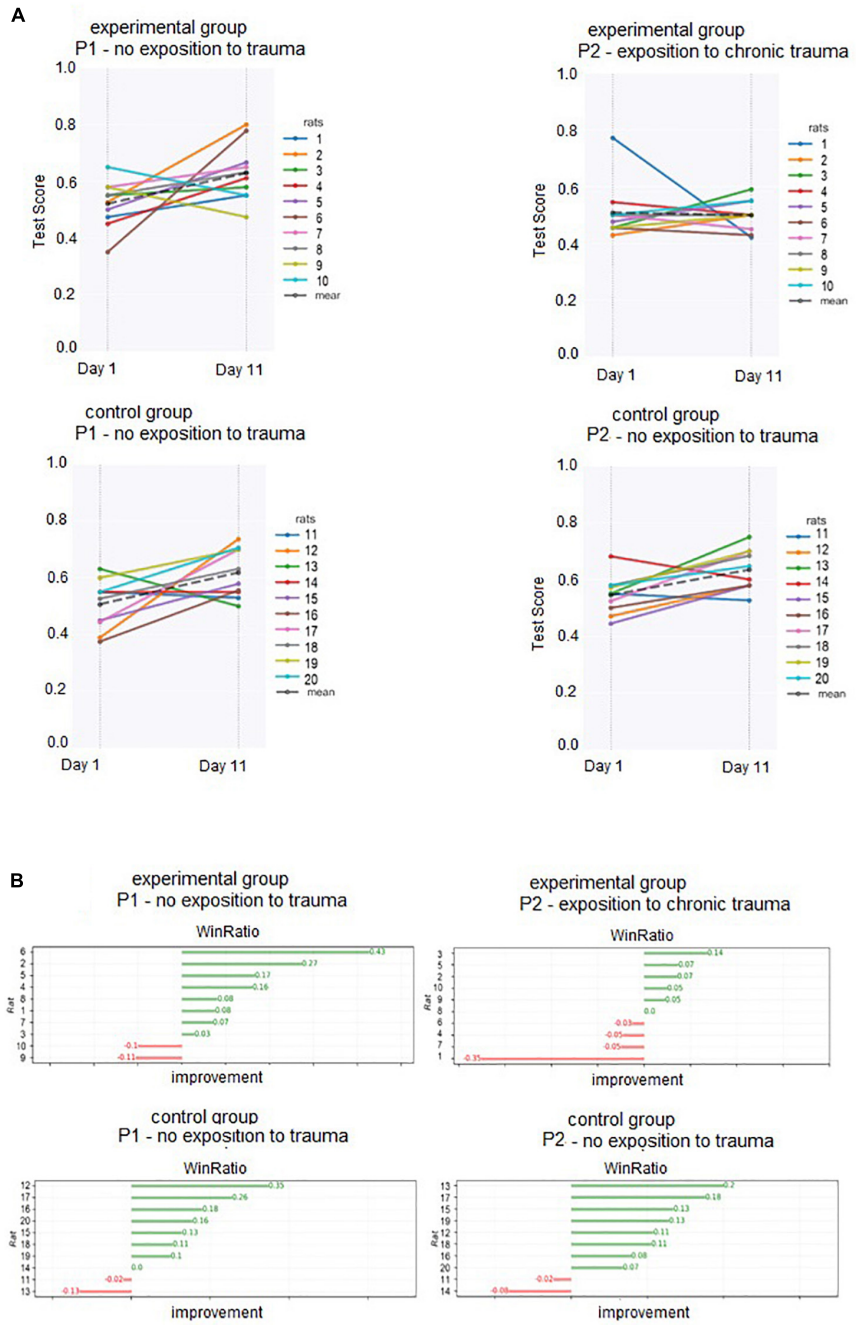

FIGURE 3 | (A) Test scores of each individual (obtained in Days 1 and 11) used to calculate P1 and P2 WinRatios. **(B)** Numerical representation of the overall WinRatio of each individual in P1 and P2.

was significantly worse than the P1 Day 11 test score (Wilcoxon Test, $p = 0.034$). In the control group, the P1 Day 12 Test score was similar to the P1 Day 11 test score (Wilcoxon two-sided, $p = 0.0557$). In the experimental group, day 12 P1 and day 12 P2 Day 12 did not differ (Wilcoxon, two sides, $p = 1.0$). In the control group, Day 12 P1 and Day 12 P2 Day 12 did not differ significantly (Wilcoxon two-sided, $p = 0.314$). In cross-group comparisons, the experimental group P1 Day 12 test score was significantly lower than in the control group (U Mann-Whitney, $p = 0.003$).

Figure 5B present differences in the time spent in an Interaction Zone of SIT in P1 and P2.The experimental group spent significantly more time in the Interaction Zone before trauma (P1) compared to time spent in Interaction Zone after predator odor (P2) (Wilcoxon two-sided test, $p = 0.019$). In the control group, there were no significant differences in the

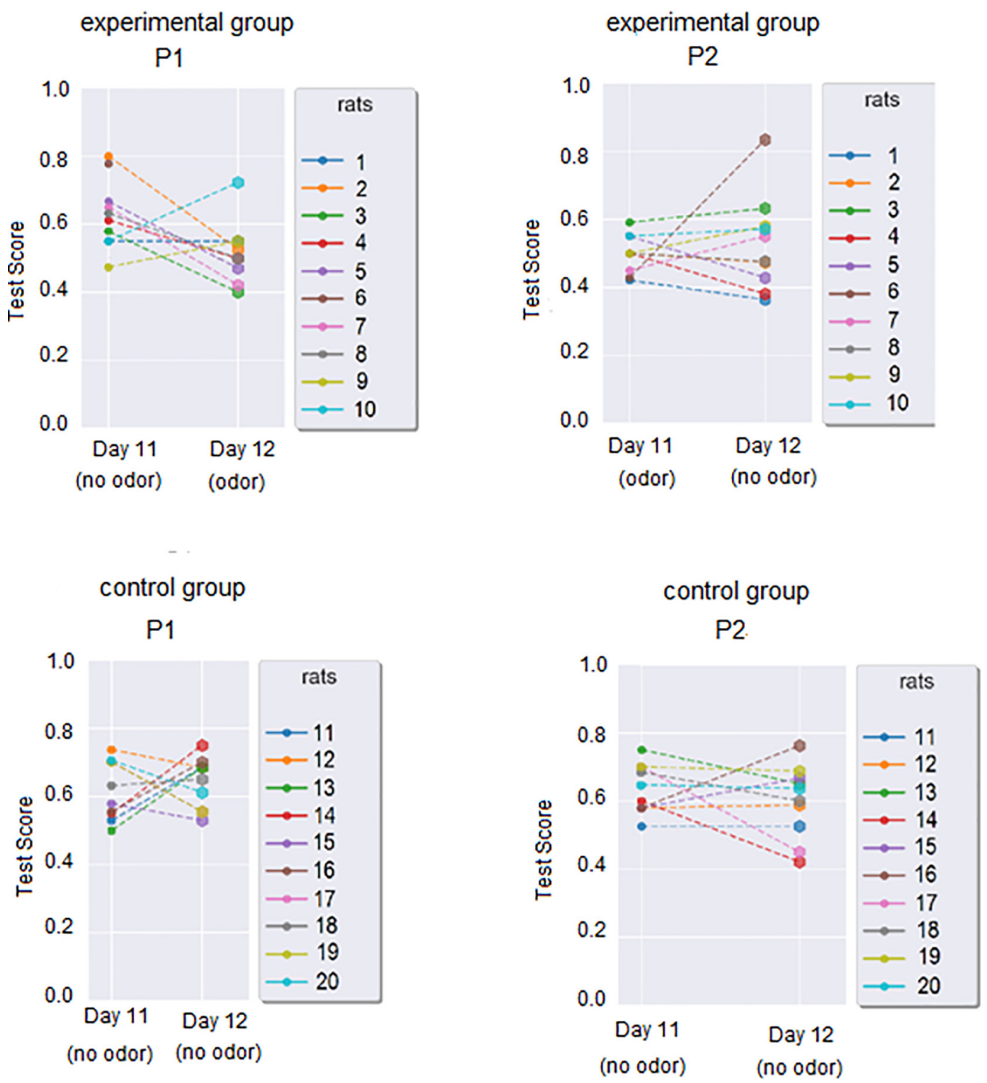

FIGURE 4 | Test scores gained during PST in the last 2 days of each phase. For the experimental group, P1 Day 11 was the day with known stimuli in PST and no odor, but the P1 Day 12 was the first exposure to odor, with stimuli known from previous days. Inversely, P2 Day 11 was the day with known stimuli in PST with odor, while P2 Day 12 was the day with known stimuli in PST, but without odor exposure.

time spent in an Interaction Zone during P1 and P2 (Wilcoxon two-sided test, $p = 0.43$). During P1, the experimental group spent similar time in an Interaction Zone to the control group (Wilcoxon two-sided test, $p = 0.038$). Similarly, cross-group comparisons did not reveal differences between both groups in time spent in an Interaction Zone during P2 (Wilcoxon two-sided test, $p = 0.91$) (see **Figure 5B**).

DISCUSSION

In our study, we examined reinforcement learning (through PST) before and after trauma and compared obtained results with the untraumatized control group. In the experimental group, exposure to chronic trauma (which occurred every day for 12 consecutive days) significantly reduced the ability to perform on PST. The decline in cognitive ability was significant immediately after the first exposure to trauma, although this result is not surprising. Previous findings indicate that single exposure to predator odor is sufficient to induce a behavioral and physiological response such as avoidance (Albrechet-Souza and Gilpin, 2019) or an increase in alcohol intake (Edwards et al., 2013). To our knowledge, we are the first to report a decline in reinforcement learning immediately after exposure to predator odor. We did not find a significant improvement in PST performance 1 day after the odor removal. This result stays in line with studies reporting that the consequences of odor exposure persist weeks after initial exposure (Albrechet-Souza and Gilpin, 2019; Schreiber et al., 2019). To the best of our knowledge, our study is the first to examine rodent cognitive abilities *via*

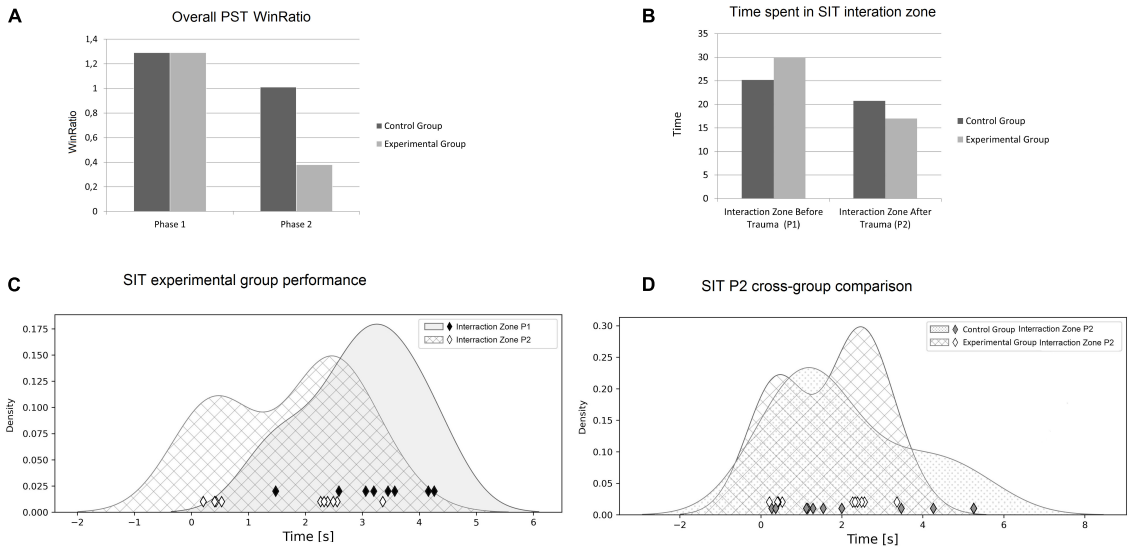

FIGURE 5 | (A) Rewards collected in PST throughout the entire experiment. **(B)** Time spent in an Interaction Zone in SIT, comparison between two groups of rats. **(C)** Time spent in an Interaction Zone during SIT (experimental group). Raw measurements data are drawn as diamonds. For easy visual groups comparison we provided kernel density estimates of Probability Density Functions (Silverman, 2018). The experimental group spent significantly more time in an Interaction Zone before trauma exposure (P1). Furthermore, exposure to trauma (P2) induced a bimodal data distribution that has not occurred in P1. **(D)** The experimental group P2 times compared to the control group P2 times. There is no significant differences in group comparisons ($p = 0.91$), but this may be due to bimodality that characterizes post stress-performance of the experimental group.

PST before and after exposure to predator odor. Moreover, our study confirmed bobcat urine utility as a traumatizing factor, as it significantly affected cognitive abilities, and influenced social behavior among rats exposed to odor.

Overall, the control group performed significantly better in P2 of the experiment. During that period of time, the experimental group was chronically exposed to predator odor. This enforced vigilance and anxiety among rats, which resulted in significant deterioration in PST performance, even though neither punishment nor the physical threat was ever delivered. There are numerous animal models with severe physical punishments, for example foot shock, underwater trauma, restrained stress (Whitaker et al., 2014). Our model is not one of them; the punishment was the lack of the reward. In humans, there are protocols that expose subjects to the possibility of punishment that is never delivered. These studies confirm that anticipation stress reduces reward sensitivity, reward responsiveness (Bogdan and Pizzagalli, 2006; Berghorst et al., 2013) and generally impairs reinforcement learning (Cavanagh et al., 2011). Interestingly, it is hypothesized that stress-susceptible individuals may be more vulnerable to punishment than reward collection (Berghorst et al., 2013). In that case, our protocol (which did not present tangible punishment) may have been less perceptive to those subjects. On the other hand, literature implies that individuals who are less stress-susceptible may be more vulnerable to reward collection than to punishment deliverance (Cavanagh et al., 2011), an observation that validates our approach. This distinction in susceptibility is discussed to be related to striatal dopamine levels, which are known to guide decision making in relation to learning from positive and negative stimuli. Patients with pharmacologically elevated dopamine levels learn better from rewards in PST, compared to those with reduced dopamine levels, who learn better to avoid punishment in PST (Frank et al., 2004). Thus, we hypothesize that the experimental group performed in PST poorer in P2, due to disrupted dopamine levels in the striatum. This implies decline in PST was related to the disruption in reward learning circuits. In humans, exposure to chronic stressors results in blunted ventral striatal (VS) neural activity during reward processing in healthy individuals (Nikolova et al., 2012), as well as in those with PTSD (Mehta et al., 2020). The prominent function of dopaminergic VS neurotransmission in reinforcement learning was confirmed in human positron emission tomography studies that mark right caudate and VS as motivational centers of engagement in activity that brings profit (Kasanova et al., 2017). Stress-related blunted dopaminergic neurotransmission results in overall worse performance in PST, a phenomenon that was observed among individuals with a familial risk of psychosis. Thus, disruption in VS is often symptomatically related to anhedonia, depression, and motivation deficits in both humans and animals (Malone et al., 2009; Roesch et al., 2009; Corral-Frías et al., 2015). We hypothesize that chronic trauma, induced in the experimental group, reduced dopamine level in VS that decreased the performance of experimental rats in PST P2. Our protocol delivered chronic trauma that compromised reward learning, but behaviorally did not induce full-blown anhedonia. We believe that is an important advantage of our model—rats perform voluntarily, which facilitates measurement of cognitive and behavioral deficits in rodents.

Our results are in agreement with studies that indicate deterioration in cognitive abilities among those exposed to

trauma. Schizophrenia patients with a history of trauma exhibit poorer cognitive functioning in terms of memory, executive functions, attention, concentration, and mental speed (Misiak et al., 2017). Computational studies present altered reinforcement learning in veterans with diagnosed PTSD, indicating alteration in reward and punishment perception and valuation (Myers et al., 2013; Brown et al., 2018). Moreover, individuals with PTSD have increased sensitivity to an unexpected outcome during PST (Brown et al., 2018). To our knowledge, this phenomenon has not been validated in animal models, although we believe our protocol may be in use in further research of this topic. If this mechanism of overreaction to an unexpected outcome occurs in the rodent model of trauma, it could have explained the deterioration in learning during P2. We hypothesize that our traumatized subjects were more susceptible to unexpected punishment in P2—as feedback was probabilistic, rewarding stimulus rarely delivered punishment. To test this hypothesis in the future, our protocol needs to be recreated using a computational model.

In SIT, the experimental group proved to be less socially oriented in P2, in comparison to P1—after trauma, rats spent less time in an Interaction Zone with an unfamiliar rat. In humans, chronic trauma influences social interactions, especially in children. Youngsters exposed to chronic traumatic stress present substantial difficulties in constructing relationships. They have troubles in interactions with other children as they often display avoidant symptoms, present inadequately sensitive flight/fight responses, respond to minor stressors by freezing (Streeck-Fischer and van der Kolk, 2000). In another study, adults with PTSD after 2-years of military deployments presented avoidance behavior, social withdrawal, had less positive engagement in relation with their families during post-deployment reengagement (Brockman et al., 2016). In rodent, chronic social defeat model reveals significant decreases in interpersonal interactions after exposure to trauma (Venzala et al., 2012). We believe results obtained during our experiment stays in line with these reports. We hypothesize that it may be related to dopamine disruption, since social behavior in rodents has been shown to be strongly dependent on neural activity in the ventral tegmental area (VTA) of the brain (Chaudhury et al., 2013). Dopamine neurons in VTA project signals to different structures in the striatum (for example, nucleus accumbens) as the well as amygdala or medial prefrontal cortex. Manipulation in neural projection dynamics of VTA influences social interactions in rodents (Gunaydin et al., 2014); therefore, we hypothesize that our trauma protocol disrupts dopamine levels in the midbrain, which results in reduced social behavior after exposition. In the control group, there was no significant difference in SIT performance in P1 and P2, as rats were not exposed to trauma. Similarly, there was no significant difference in the performance of the experimental group P1 and the control group P1 in SIT, as none of the subjects was exposed to predator odor. Although the experimental group spent significantly more time in an Interaction Zone during P1 in comparison with P2, statistical analysis does not reveal differences in time spent in an Interaction Zone between experimental group and the control during P2. This result is inconclusive—two factors have to be taken into consideration. First, a performance difference was observed (see **Figure 5B**), but we cannot support this with statistical verification, probably due to the small number of rats tested. Second, the distribution of the time spent in an Interaction Zone among rats exposed to trauma was bimodal (see **Figure 5C**). This makes the verification of this particular result ambiguous, as a control group did not present this tendency (see **Figure 5D**). This may be a random result, as the group was small in number, but it may also be hypothesized that exposition to trauma divided the experimental group into two subgroups; individuals more susceptible to chronic trauma (less time in an Interaction Zone) and those more resilient (more time in an Interaction Zone). This requires further verification with a larger group, but if confirmed, that would imply that SIT shows individual variability in reactivity to stress induced by predator odor.

Limitations

There are components of our research that should be expanded. As discussed earlier, a categorization is often applied in human studies of the subject, where individuals are characterized as stress-susceptible or resilient. We believe that our protocol could benefit if such a distinction was applied. A viable possibility may be the Avoiders/Non-Avoiders distinction proposed by Albrechet-Souza and Gilpin (2019) in their animal model of PTSD, or a hypothesized distinction delivered by SIT, as we discussed in paragraph above. While rats were in PST chambers, we did not videotape their activity. This is why we could not provide behavioral data from that time-period, that might have been interesting. Our conclusions regarding dopamine-related VTA and VS activity need further verification by molecular studies in animal models. Furthermore, there are interesting reports on striatal activity heavily influenced by increased inflammatory biomarkers, in the context of trauma (Mehta et al., 2020). We believe that our protocol could be of use in further exploration of these topics.

We believe further studies with our protocol should apply an additional group of rats exposed to non-predator odor. This could validate our approach with bobcat urine as a stressor, and deliver much needed comparative context. Changes in rodent behavior could be explored in exposure to different odors, for example alpha-pinene or green leaf odor that are known to have stress-alleviating effects (Akutsu et al., 2003). Studies that use different odors to examine behavioral and cognitive changes are sparse, thus we hypothesize our protocol could be of use to study this subject. We believe this comparative context would deliver interesting results in the wide issue of rodents behavioral and cognitive performance analysis.

CONCLUSION

We present our protocol that may be useful in assessing cognitive abilities in rodents. Rats performed PST voluntarily, when exposed to chronic trauma induced by predator odor. Performance in PST was measured before and after trauma in the same group of rats. Subjects obtained better results in PST before exposure to predator odor. Overall, the experimental group

scored lower in PST compared to not-traumatized control. After exposure to chronic trauma, rats were less socially oriented in SIT, compared to the results obtained before the trauma protocol. Moreover, traumatized rats presented a bimodal tendency in time spent in an Interaction Zone with unknown rat, but due to a small number of animals tested, this result needs further verification.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Ethics Committee for Animal Experiments, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

AUTHOR CONTRIBUTIONS

TB designed the research, carried out laboratory experiments, and wrote the manuscript with input from all authors. PK designed and constructed Testing Chambers. JD performed mathematical calculations and analyzed obtained data. BS delivered theoretical framework. DF provided critical feedback and helped shape the research.

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Neuroligin Plays a Role in Ethanol-Induced Disruption of Memory and Corresponding Modulation of Glutamate Receptor Expression

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Exposure to alcohol causes deficits in long-term memory formation across species. Using a long-term habituation memory assay in *Caenorhabditis elegans*, the effects of ethanol on long-term memory (> 24 h) for habituation were investigated. An impairment in long-term memory was observed when animals were trained in the presence of ethanol. Cues of internal state or training context during testing did not restore memory. Ethanol exposure during training also interfered with the downregulation of AMPA/KA-type glutamate receptor subunit (GLR-1) punctal expression previously associated with long-term memory for habituation in *C. elegans*. Interestingly, ethanol exposure alone had the opposite effect, increasing GLR-1::GFP punctal expression. Worms with a mutation in the *C. elegans* ortholog of vertebrate neuroligins (*nlg-1*) were resistant to the effects of ethanol on memory, as they displayed both GLR-1::GFP downregulation and long-term memory for habituation after training in the presence of ethanol. These findings provide insights into the molecular mechanisms through which alcohol consumption impacts memory.

Keywords: ethanol, *C. elegans*, glutamate receptor, neuroligin, memory blackout

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INTRODUCTION

The consequences of alcohol consumption in humans include deficits in decision-making, problem-solving, and in learning and memory (Leckliter and Matarazzo, 1989; Selby and Azrin, 1998). Alcohol intoxicated individuals show impaired performance on tasks such as learning word lists (Grant, 1987), short- and long-term logical memory (Selby and Azrin, 1998), and general working memory (Ambrose et al., 2001). Further, people who have experienced alcohol-induced blackouts continue to show impaired recall the next day when sober (Jackson et al., 2021). At the neuron level, cellular correlates of memory (primarily long-term potentiation and depression) are both attenuated by ethanol exposure (White et al., 2000; Chandler, 2003; Izumi et al., 2005; Avchalumov and Mandyam, 2020; Mira et al., 2020). Further, chronic alcohol exposure reportedly causes significant changes in expression of the overall brain transcriptome in prefrontal cortex neurons of mice (Liu et al., 2022). Although many studies have identified neural effects of alcohol consumption, the mechanism(s) by which alcohol affects learning and memory still requires investigation.

Ethanol has been found to interact with several neurotransmitter systems. The role of GABA in mediating the intoxication effect of ethanol are well-established (see Kumar et al., 2009; Chandler et al., 2017). Glutamate has also been shown to be affected by ethanol (see Chastain, 2006; Rao et al., 2015). A number of correlations have been reported between ethanol-induced changes in glutamate receptor activity and the behavioral effects of ethanol (i.e., Harris et al., 1998; Chandler, 2003; see Valenzuela, 1997, or Woodward Hopf and Mangieri, 2018). Gioia and McCool (2017) reported an inhibitory effect of ethanol on neurons of the basolateral amygdala in mice, an area involved in fear conditioning. Gioia et al. (2017) found that ethanol had a negative effect on vesicle recycling proteins at glutamatergic synapses in part due to an α-amino-3-hydroxy-5-methyl-4-isooxazole (AMPA) receptor-mediated form of post-synaptic facilitation. As well, Salling et al. (2016) showed that animals that consumed ethanol showed increased AMPA receptor expression in the central amygdala suggesting some of the effects of ethanol on learning and memory may be mediated by modulation of glutamate signaling.

Ethanol affects behaviors across a variety of species and may do so through orthologous gene pathways (Crabbe et al., 1994). Given the effects of ethanol on memory and on glutamate signaling reported in mammalian brains, the current study investigated the effects of ethanol on long-term habituation, a glutamate-dependent form of long-term memory in the *C. elegans* model system (Rose et al., 2002, 2003). Habituation is a non-associative form of learning that is observed as a decrease in response to repeated stimulation. Long-term memory (>24 h) for habituation is seen following a spaced training protocol (Rose and Rankin, 2001). This long-term memory for habituation is glutamate-dependent and is correlated with decreased punctal expression of GLR-1, an AMPA/KA-type glutamate receptor subunit (Rose et al., 2003). The current study examined whether ethanol exposure during training would impair long-term memory and block the decrease in GLR-1 punctal expression levels after training. A surprising discovery that GLR-1 punctal expression is increased in untrained animals exposed to ethanol led to investigation of a possible role of the postsynaptic cell adhesion protein neuroligin (NLG-1). Results indicate that ethanol exposure during training disrupts the formation of long-term memory, and that NLG-1-mediated regulation of GLR-1 levels may underlie the memory deficits observed.

METHODS

Animals

Worms were maintained on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* (OP50) and maintained at 20°C. *C. elegans* wild-type N2 Bristol and *nlg-1*(ok259) strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). KP1580 *pglr-1*::GLR-1::GFP was provided by J. Kaplan (Harvard University, Boston, MA). RM3389 *nlg-1*(ok259); p*nlg-1*::NLG-1::YFP was provided by J. Rand (Oklahoma Medical Research Foundation, OK).

Ethanol Plate Preparation

To ensure consistent agar conditions across days, single plates were weighed on training day. The appropriate amount of ethanol (100%) was added to the agar for each plate to the desired concentration (0.2 M, 0.4 M, or 0.6 M), then plates were wrapped in parafilm for 1–2 h to allow equilibration of ethanol into the agar. Previous work using gas chromatography found that 0.5 M ethanol exposure in *C. elegans* corresponds to a clinically relevant concentration of approximately 0.3–0.4% blood alcohol concentration (Alaimo et al., 2012). Ethanol concentrations at this level remain low enough to effectively measure behavior without producing immobility (Davies et al., 2003; Alaimo et al., 2012). Testing plates were seeded with E. coli 1 day prior to testing.

Behavior Testing and Analysis

Behavioral observations of small groups of worms were made using a Wild Leitz stereomicroscope (Zeiss, Canada). Petri plates containing 15–20 worms received a tap (∼1.5 N force) delivered to the side of the Petri plate via a copper rod connected to a 6-V electromagnetic relay. This "tapper" was activated with a Grass S88 (Quincy, MA) stimulator set to deliver a single tap stimulus. Worm locomotor responses were video recorded for analysis. The reversal response magnitude (i.e., the distance the worm crawled backwards in response to the tap stimulus) was scored using stop-frame video analysis. Response tracings were quantified in NIH Image (version 1.63). Two-way mixed ANOVAs with Fisher's protected least significant difference (PLSD) *post-hoc* tests were used to test for statistical significance. If only two groups were analyzed, then student *t*-tests were employed.

Long-Term Habituation Training and Testing

Four-day-old worms (∼90–96 h after eggs were laid at 20°C) were transferred to either ethanol-containing plates or ethanol-free training plates (∼20 worms/plate) and allowed 1 h to recover from transfer and to absorb ethanol. To deliver uniform mechanosensory stimuli to a large number of training plates simultaneously, training plates were placed in a Tupperware container and dropped from a height of ∼5 cm onto a tabletop. Training consisted of four blocks (20 drops given at a 60 s interstimulus interval, ISI) with training blocks separated by 1-h rest periods. Untrained groups were dropped once immediately at the end of the trained groups to control for any potential influence of pseudoconditioning due to responding to novelty. One hour after training, all worms were transferred to ethanol-free plates containing a small amount of OP50 *E. coli* (50–100 μL). For conditions where worms were exposed to ethanol for seven hours after training, both trained and untrained worms were transferred to fresh ethanol plates 1 h after training and to ethanol-free plates at the end of the ethanol exposure time.

To test retention, 24 h after habituation training 15–20 worms (5-days-old from egg-laying) were transferred to either a plain agar plate (normal long-term retention) or a plate containing ethanol (0.4 M to test for state conditioning and 0.05 M for

context conditioning) and given 1 h to acclimate. After a 6-min pretest period, worms were given five tap stimuli at a 60 s ISI and the responses were recorded. Prior to each tap stimulus, plates were repositioned on the microscope stage to maximize the number of worms in the field of view meaning that responses were captured as a population measure, randomized across worms.

Testing Short-Term Habituation

Four-day-old worms were transferred to a test plate containing 0.0 M, 0.2 M or 0.4 M ethanol. A tap stimulus was delivered to the side of the plate either at a 10 s ISI or a 60 s ISI and worm locomotor responses were video recorded and analyzed (see Behavior Testing and Analysis).

Confocal Fluorescence Imaging

To quantify levels of GLR-1::GFP punctal expression, worms were paralyzed in 12 μL of 50 mM sodium azide solution on a sterile glass microscope slide. Worms were then placed on a 2% agar pad and covered with a 1.5 thickness glass coverslip for imaging. Images of GLR-1::GFP clusters along the ventral nerve cord were collected with an Olympus FV1000 confocal microscope (Leica SP8 for the experiments examining effects of ethanol only on GLR-1 expression and **Supplementary Figure 1**) with optical sections collected at 0.5 μm intervals using a 63× oil immersion lens and consistent confocal microscope settings: gain = ∼1.0; PMT = 600 (+/−50), laser = ∼1.0%. FIJI and ImageJ v1.33 were used to measure area of fluorescence punctal expression from Z-projected image stacks.

RNA Isolation and Quantification

C. elegans RNA was isolated using Trizol extraction (two biological replicates at three dilutions performed in triplicate for each group). RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR with oligo(dT) primers according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The ΔΔCT method of quantification was performed on an ABI 7000 (Applied Biosystems, Foster City, CA) using SYBR GREEN. For *glr-1* and *act-1* (reference gene) the following primers were employed: forward GGA GAG GTT CTG GTT TTGATT GA and reverse TCG AGT ACG AAG ATG TCT CCA AAG for *glr-1*; forward TTG CCC CAT CAACCA TGA A and reverse CTC CGA TCC AGA CGG AGT ACT T for *act-1* (Ebrahimi and Rankin, 2007).

RESULTS

Ethanol Exposure During Training Blocks Long-Term Memory

To investigate whether long-term memory for habituation could be disrupted by ethanol in a dose-dependent manner, wild-type worms were trained on agar plates infused with 0.2 M, 0.4 M or 0.6 M ethanol (as previously described in Davies et al., 2003) and tested for memory 24 h later (**Figure 1A**). Overall, there was a significant interaction between training and ethanol exposure ($F_{7,332} = 2.603$, $p < 0.05$). Wild-type worms not exposed to ethanol showed long-term memory as evidenced by significantly smaller reversal responses to the five test taps delivered 24 h after training. Although naïve worms exposed to 0.2 M or 0.4 M ethanol showed a small decrease in reversal magnitude compared to naïve worms not exposed to ethanol, only the worms trained on 0.2 M ethanol showed significant long-term memory 24 h after training ($p < 0.05$). Worms that were trained at higher ethanol concentrations (0.4 M and 0.6 M) showed no evidence of memory for the training ($p > 0.10$). This memory deficit at higher ethanol concentrations was not the result of differences in food quality (ethanol can act as a bactericide for the *C. elegans* OP50 bacterial food source at higher concentrations) as worms tested either "on" or "off" food still showed a significant effect of training ($F_{3,154} = 8.840$, $p < 0.001$; **Supplementary Figure 2**).

To test whether the effects of alcohol on memory are specific to exposure during training rather than a generalized memory impairment, worms were first trained in the absence of ethanol, then 1 h after training were exposed to 0.4 M ethanol for a 7-h period (corresponding to the duration of ethanol exposure in the previous experiment). Worms were then tested 24 h after the end of the ethanol exposure (**Figure 1B**). In this condition, worms exposed to ethanol after training showed a decrement in response to the test taps compared to untrained groups ($F_{3,204} = 3.201$, $p < 0.05$; **Figure 1B**) suggesting that alcohol-induced memory impairment is specific to ethanol exposure during training.

Worms were tested in two cued conditions (context and state conditioning) to determine whether the memory-impairing effect of alcohol during training is the result of a retrieval deficit. Previous research from our lab showed that context conditioning enhances retention of habituation when worms are trained and tested in the presence of the same chemosensory cue (Rankin, 2000; Lau et al., 2013). We tested whether a contextual cue could restore memory for training delivered in the presence of ethanol by testing in the presence of a subtle ethanol cue (0.05 M ethanol; a dose strong enough for the worms to detect the ethanol, but not strong enough to induce an intoxicated state; Bettinger and McIntire, 2004). Alternatively, memory for sensory adaptation training in *C. elegans* that occurred in the presence of ethanol could also be state-dependent (Bettinger and McIntire, 2004). In a *Drosophila* chronic alcohol exposure paradigm, flies only remembered to move away from an attractive odor previously paired with heat shock if they were given a high dose of alcohol again (Robinson et al., 2012). To test whether an inebriated state would cue recall and restore memory, worms were re-exposed to the same ethanol concentration (0.4 M) delivered at training during testing. Memory was only observed in the untreated group in worms that were not exposed to ethanol during testing ($F_{5,378} = 2.813$, $p < 0.05$; **Figure 1C**). Thus, neither the contextual cue nor state-dependent recall was sufficient to restore memory for training that occurred in the presence of ethanol, suggesting that the memory deficit seen with ethanol exposure during training was due to impairments on encoding.

Short-Term Habituation Affected by Alcohol When Stimuli Presented at Long Interstimulus Intervals

It is possible that the effects of alcohol on memory for habituation were due to a short-term memory and/or habituation learning

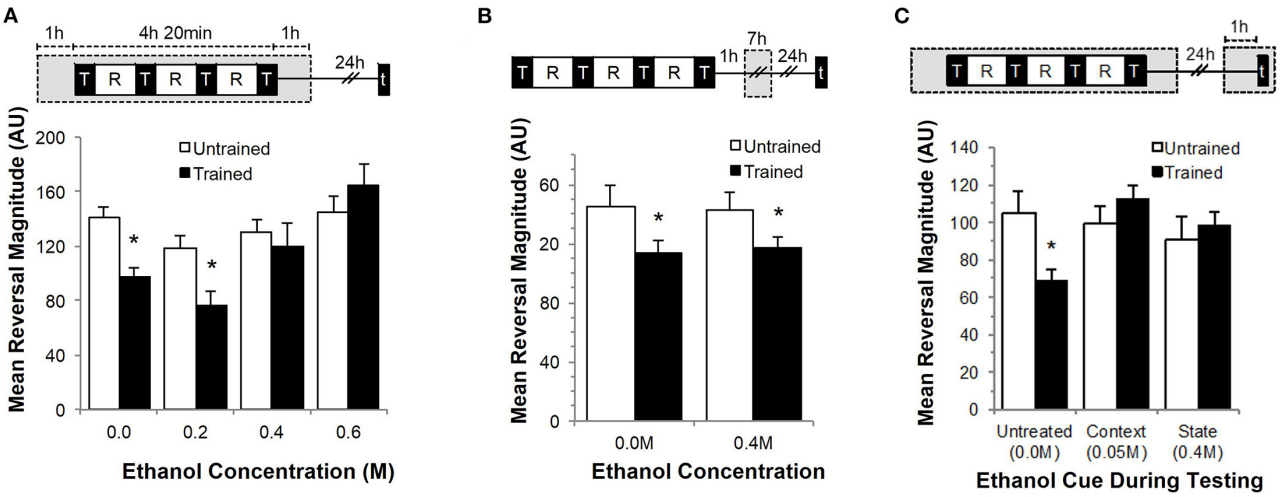

FIGURE 1 | Effects of ethanol exposure during training on long-term memory for habituation. Each graph includes an illustration of corresponding training and ethanol exposure protocols where gray boxes indicate time of ethanol exposure while black boxes indicate training periods denoted by "T" and test periods denoted by "t." White squares indicate rest periods denoted by "R." **(A)** Mean reversal response magnitude (± SEM) measured at testing 24 h after exposure to 0.0, 0.2, 0.4 or 0.6 M ethanol exposure compared between trained (black bars) and untrained groups (white bars). **(B)** Mean reversal response magnitude (± SEM) compared between trained (black bars) and untrained groups (white bars) with 7-h ethanol exposure delivered 1-h after training completed. **(C)** Mean reversal response magnitude (± SEM) measured after 0.4 M ethanol exposure during training and re-exposed to either 0.05 M (context cue) or 0.4 M (internal state cue) at testing compared to an unexposed conditioned group. * = $p < 0.05$.

impairment. To assess this, worms were given 30 tap stimuli at either a 10 s or a 60 s ISI and the reversal response magnitude was measured for each stimulus presentation to test whether short-term habituation was affected by alcohol. When stimuli were delivered at a 10 s ISI, exposure to 0.4 M ethanol resulted in no significant difference between the untreated control group and the ethanol-treated group in the rate of habituation or the final asymptotic habituated response level (**Figure 2A**; $F_{9,36} = 0.537$, $p > 0.10$).

However, when stimuli were delivered at a longer 60 s ISI, there were significant decreases in habituation between the untreated group and both the 0.2 M and the 0.4 M ethanol treated groups ($p < 0.05$ and $p < 0.01$, respectively). At this longer 60 s ISI the two-way ANOVA revealed a small but significant effect of ethanol exposure ($F_{18,378} = 1.947$, $p < 0.05$; **Figure 2B**). Interestingly, there was only a significant difference between the untreated and 0.4 M ethanol treated groups for asymptotic level of response magnitude ($p < 0.05$), and not between the untreated control and the 0.2 M ethanol treated group. Taken together this data indicates that alcohol exposure does not affect short-term habituation at 10 ISI, but decreases habituation levels at a longer 60 s ISI in both the 0.2 M and 0.4 M ethanol conditions. Despite decreasing 60 s ISI tap habituation levels at 0.2 M, the previous long-term memory experiment (**Figure 1A**) showed that animals trained in 0.2 M ethanol still exhibited long-term memory. This suggests that the mild impairment effects of alcohol exposure on short-term 60 s ISI habituation does not impede the formation of long-term memory.

Ethanol During Training Interferes With Decreased Glutamate Receptor Expression Seen With Long-Term Memory for Habituation

The long-term memory for habituation training protocol used here has been shown to lead to decreased punctal expression of GLR-1, an AMPA/Kainate type glutamate receptor subunit homologous to mammalian GluRs (Rose et al., 2003; Rose and Rankin, 2006). In *C. elegans*, GLR-1::GFP transgene expression in the ventral nerve cord is generally seen as bright puncta representing mostly synapses (Hart et al., 1995). To determine whether alcohol would alter this training-dependent decrease in GLR-1, punctal expression levels of GLR-1::GFP were measured 24 h after training on 0.0 M or 0.4 M ethanol (**Figure 3A**). Indeed, there was a significant difference in punctal expression levels between ethanol exposure groups ($F_{3,64} = 5.179$, $p < 0.01$; **Figure 3B**). GLR-1::GFP levels for the no-ethanol group were consistent with previous findings: worms trained 24 h earlier without ethanol (0.0 M group) showed a significant decrease in total area of GLR-1::GFP punctal expression in the ventral cord compared to untrained worms ($p < 0.05$). By comparison, when exposed to 0.4 M ethanol during training, GLR-1::GFP punctal expression levels for the untrained and trained groups were not significantly different ($p > 0.10$), consistent with observations from our behavioral studies that there is a deficit in long-term memory for training in these animals. Interestingly, although GLR-1::GFP punctal expression levels from worms trained on 0.4 M ethanol did not show a training-dependent decrease in GLR-1::GFP punctal expression level, the untrained

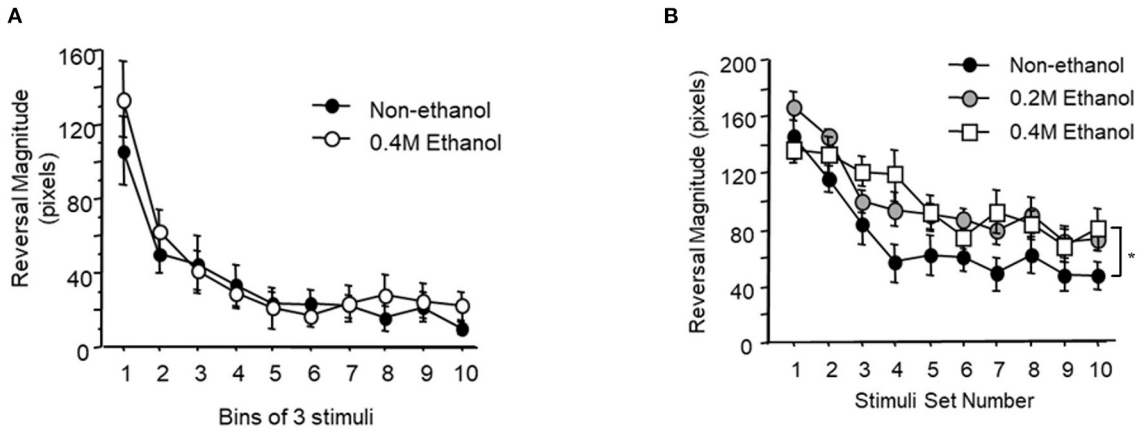

FIGURE 2 | Short-term habituation during ethanol exposure. **(A)** Mean reversal magnitude (±SEM) to 30 tap stimuli delivered at a 10 s interstimulus interval and averaged in bins of three stimuli for control (black circle) vs. worms exposed to 0.4 M ethanol (white circle). **(B)** Mean reversal magnitude (±SEM) to 30 tap stimuli delivered at a 60 s ISI averaged in bins of three stimuli for control (black circle), 0.2 M ethanol exposed worms (gray circle) and 0.4 M ethanol exposed worms (white square).

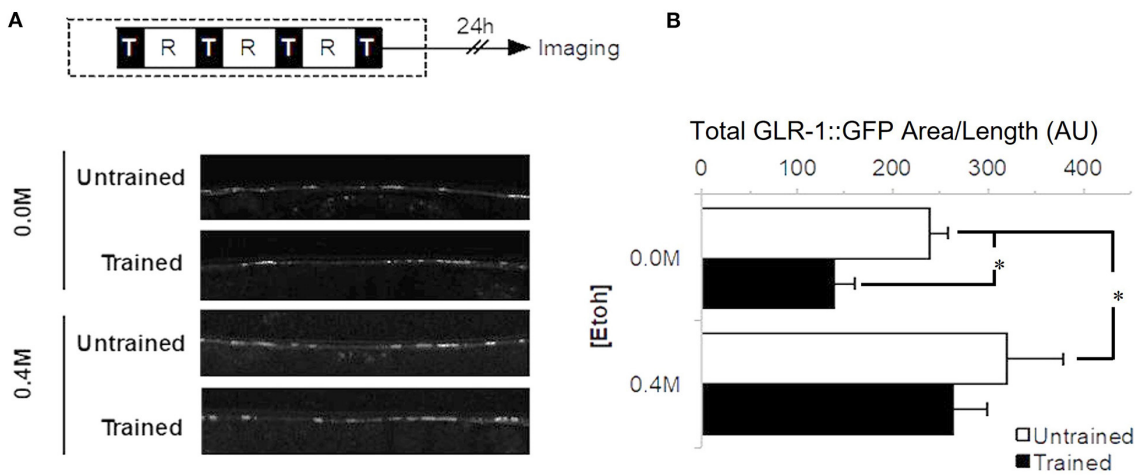

FIGURE 3 | Expression of GLR-1 receptors 24 h after long-term memory training. **(A)** Behavioral protocol whereby long-term memory training is delivered in four blocks (black squares with "T") separated by 1-h rest periods (white square with "R"). Gray shaded area corresponded to timing of ethanol exposure. Examples of confocal images of GLR-1::GFP expression captured from worms exposed to 0.4 M ethanol, comparing punctal expression levels between trained and untrained. **(B)** GLR-1::GFP punctal expression measured as Mean Total GFP expressing area/worm (±SEM) with measures normalized by distance measured along the ventral nerve cord. Measures captured between control ($n = 15$) and trained worms (0.0 M ethanol; $n = 18$) as well as control ($n = 17$) and trained ($n = 18$) worms on 0.4 M ethanol with comparisons between trained (black bars) and untrained groups (white bars). * = $p < 0.05$.

ethanol exposure group showed a significant increase in GLR-1::GFP punctal expression compared to the 0.0 M control group ($p < 0.05$).

Alcohol-Induced Long-Term Memory Impairment Requires NLGN1

In mammals, AMPA receptor trafficking and recruitment involves a postsynaptic cell adhesion protein, neuroligin-1 (NLGN1; Heine et al., 2008; Mondin et al., 2011). NLGN1 has been shown to be involved in stabilizing AMPA receptors at synapses, and this process can occur within 2–8 h (Zeidan and Ziv, 2012). NLGN1 also binds to the post-synaptic density complex PSD-95 to interact with it and other proteins, and has been shown to play an integral role in controlling the function of excitatory synapses through AMPAR regulation (Nam and Chen, 2005). Interestingly, ethanol has also been shown to influence post-synaptic density of glutamatergic receptors (Chandler, 2003; Burnett et al., 2016). Based on these observations, we hypothesized that neuroligin might play a role in mediating the effects of ethanol on long term memory for habituation.

The *C. elegans* gene *nlg-1* encodes the worm ortholog of NLGN1 (Hunter et al., 2010). To test whether *C.*

elegans neuroligin played a role in the effects of ethanol on long-term memory for habituation in *C. elegans*, we first measured reversal responses of *nlg-1*(ok259) loss of function deletion worms, 24 h after they were given long-term habituation training either on or off 0.4 M ethanol (**Figure 4A**). As expected, wild-type worms showed memory when trained in the absence of ethanol, but not when trained on 0.4 M ethanol (**Figure 4B**). Interestingly, *nlg-1*(ok259) worms that had received training had significantly smaller reversal responses to test taps compared to untrained worms when trained either on or off of 0.4 M ethanol ($F_{7,529} = 6.622$, $p < 0.01$; **Figure 4C**) suggesting that alcohol did not disrupt memory in the absence of a functional *nlg-1* gene.

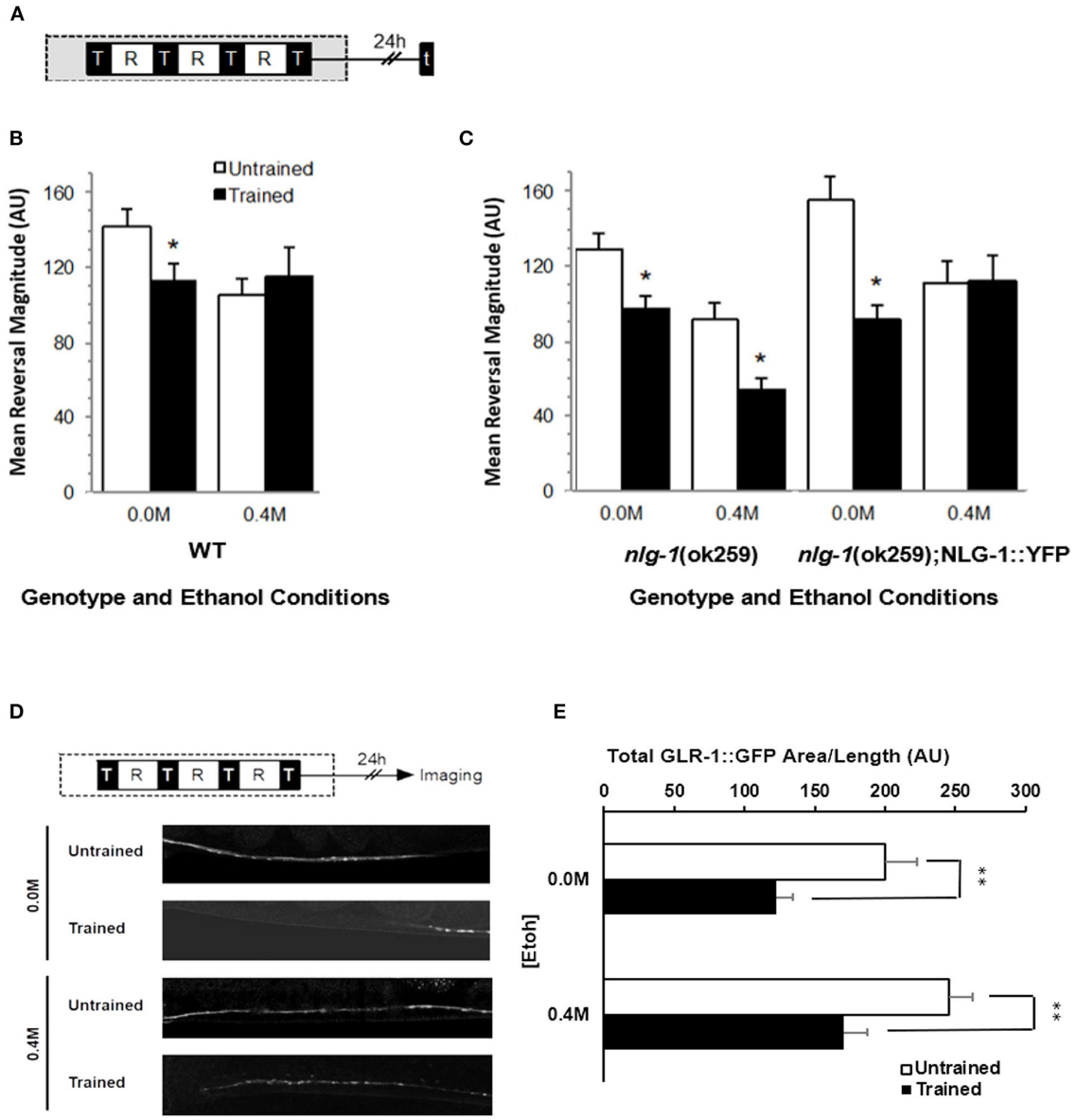

FIGURE 4 | Effects of ethanol exposure during training on long-term memory in *nlg-1* mutant worms. **(A)** Long-term habituation memory training protocol with four training blocks (black squares marked by "T") separated by 1-h rest period (white squares marked by "R"). 5 test-tap trial indicated by black square marked with "t" or GFP "imaging" was performed 24 h after training. Gray shaded area indicates period of ethanol exposure. **(B)** Mean reversal response magnitude (± SEM) to 5 test taps for trained groups (black bars) compared to untrained groups (white bars) for wild-type worms either on (0.4 M) or off (0.0 M) ethanol. **(C)** Mean reversal response magnitude (± SEM) across 5 taps of *nlg-1* mutants or *nlg-1* mutant worms expressing an NLG-1 rescue transgene. **(D)** Examples of confocal images of GLR-1::GFP punctal expression captured from *nlg-1* background worms exposed to 0.4 M ethanol, comparing expression levels between trained and untrained. **(E)** GLR-1::GFP punctal expression measured as Mean Total GFP expressing area/worm (±SEM) with measures normalized by distance measured along the ventral nerve cord. Measures captured between control (0.0 M) and 0.4 M ethanol exposure groups with comparisons between trained (black bars) and untrained groups (white bars). * = $p < 0.05$ ** = $p < 0.01$.

This result was not due to a difference in ethanol sensitivity between wild-type and *nlg-1*(ok259) worms as locomotor speed, an indicator of drug tolerance in *C. elegans* (Davies et al., 2004), showed no difference in the presence of ethanol (**Supplementary Figure 3**).

To confirm that *nlg-1* expression was necessary for the ethanol-induced disruption of memory, a *nlg-1* rescue strain was also tested in which an integrated wild-type copy of *nlg-1* fused with YFP driven by the *nlg-1* endogenous promoter was expressed in the *nlg-1* mutant strain (*nlg-1*(ok259); $_{p}$*nlg-1*::NLG-1::YFP). *nlg-1* rescue worms trained without ethanol showed significantly smaller responses to test taps indicative of memory similar to wild-type worms ($p < 0.05$), and *nlg-1* rescue worms trained on ethanol did not show long-term memory ($p > 0.10$; **Figure 4C**).

To support the behavioral findings, GLR-1::GFP punctal expression in *nlg-1* mutant worms was imaged 24 h after long-term habituation training and analyzed (**Figure 4D**). In these conditions, *nlg-1* worms showed a restored decrement in GLR-1::GFP punctal expression when trained in the presence of ethanol ($p < 0.01$), similar to GLR-1::GFP punctal expression in trained WT worms ($p < 0.01$; **Figure 4E**). This result indicates that the *nlg-1*(ok259) mutation seems to preserve the learning-induced decrease in GLR-1::GFP punctal expression, a decrease that appeared to be inhibited by ethanol (**Figure 3B**). Furthermore, there was no statistical difference in GLR-1::GFP punctal expression levels between untrained *nlg-1* or WT background worms in the presence of ethanol ($p > 0.15$). These data confirm an important role for NLG-1 in mediating the negative effects of ethanol on long-term memory for habituation and the corresponding decrease in GLR-1 punctal expression in *C. elegans*.

Neuroligin (NLG-1) Is Necessary for the Alcohol-Induced Increase in Glutamate Receptor Expression

Because we found that in wild-type worms exposure to ethanol led to an increase in GLR-1::GFP 24 h following exposure to ethanol (**Figure 3**), we further investigated the characteristics of this increase by measuring GLR-1::GFP punctal expression in WT and *nlg-1* mutant worms 1 h after a 7-h exposure to 0.4 M ethanol (corresponding to the duration of ethanol exposure during long-term memory training in earlier experiments). GLR-1::GFP punctal expression in the *nlg-1* mutant worms appeared as bright puncta in the ventral nerve cord similar to that seen in wild-type worms (**Figure 5A**).

We found that 1 h after a 7-h 0.4 M ethanol exposure GLR-1::GFP punctal expression increased significantly compared to 0.0 M exposure in wild-type worms ($F_{1,43} = 4.941$, $p < 0.05$; **Figure 5B**); this increase persisted for at least 24 h (**Supplementary Figure 1**). Interestingly, there were no significant differences in GLR-1::GFP punctal expression levels of *nlg-1* mutant worms between the 0.0 M and the 0.4 M ethanol groups ($t_{24} = -0.022$, $p =$ N.S.; **Figure 5B**). In contrast, in *nlg-1* worms there were no significant differences between 0.0 and 0.4 M ethanol exposure groups in GLR-1::GFP punctal expression levels 7 or 24 h after ethanol exposure. These data suggest that functional neuroligin plays an important role in the ethanol-induced increase in GLR-1 expression seen in wild-type worms. The increase in GLR-1::GFP expression in wild-type worms after

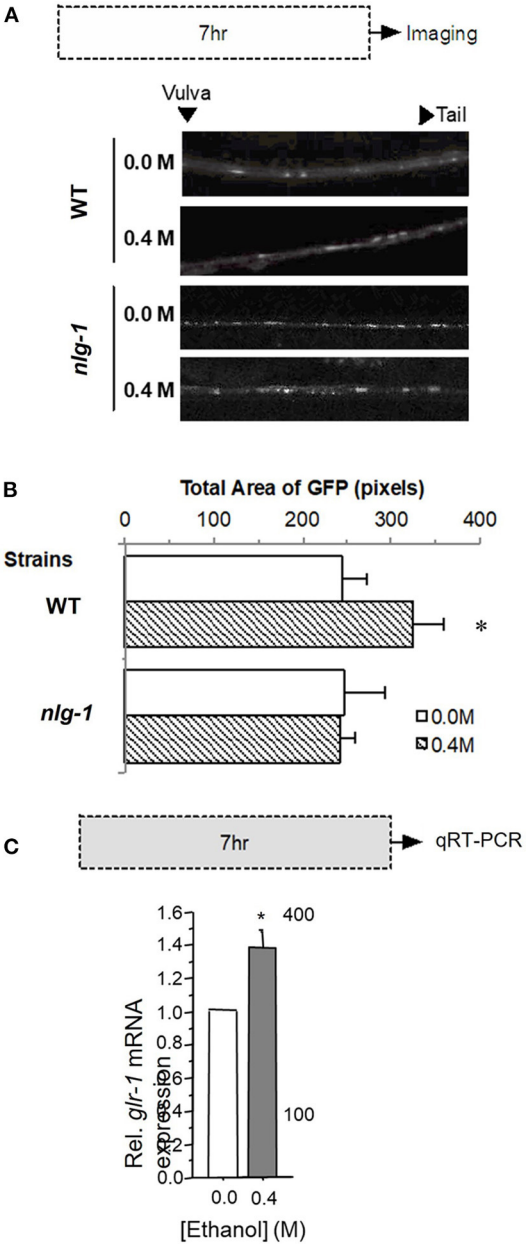

FIGURE 5 | Expression of GLR-1 receptors in either a wild-type or *nlg-1* mutant worm background after 7 h ethanol exposure. **(A)** Protocol for 0.4 M ethanol exposure (gray rectangle) followed by confocal imaging and sample images captured from GLR-1::GFP worms that are either WT (for *nlg-1*) or carry a mutation in *nlg-1* exposed to either 0.0 or 0.4 M ethanol. **(B)** Mean Total GLR-1::GFP area (± SEM) for WT vs. *nlg-1* mutant worms exposed to either 0.0 or 0.4 M ethanol following 7-h exposure period. **(C)** *glr-1* mRNA levels relative to actin (*act-1*) expression (± SD) in control worms (0.0 M ethanol) compared to treatment (0.4 M ethanol) following 7-h exposure period. * = $p < 0.05$.

the 7-h ethanol exposure was confirmed with qRT-PCR which showed that glr-1 mRNA expression was 1.4 fold higher in 0.4 M ethanol treated worms than in untreated controls ($p < 0.05$; **Figure 5C**).

DISCUSSION

Our findings indicate that ethanol exposure during training interferes with long- term memory for that training. Consistent with alcohol-induced memory impairments in a number of other species including humans, we found that the effect of alcohol on memory was dose-dependent as exposure to higher concentrations of ethanol during training effectively blocked memory 24 h later (**Figure 1A**). Worms on a 0.4 M ethanol plate for an hour prior to training would reach the equivalent of ∼0.3 percent blood alcohol concentration (BAC) estimated from internal concentration (Alaimo et al., 2012). In humans this concentration would be equivalent to consuming 6–8 drinks of alcohol, and would cause severe physical and sensory impairment. Interestingly, laboratory studies with humans on the effect of acute alcohol consumption on alcohol-induced memory blackouts by Goodwin et al. (1970) found that average peak blood alcohol concentration of individuals who showed memory disruption for events that occurred while drinking was ∼0.28 percent. Therefore, the higher ethanol concentration reported here (0.4 M) should produce a sufficient level of intoxication similar to humans.

In *C. elegans* the ethanol-induced memory impairment was specific for events that occurred during ethanol exposure as ethanol given after training had no effect on memory (**Figure 1B**). Finally, the current study found that neither context nor internal state cues present during testing could restore memory (**Figure 1C**), suggesting a deficit in memory encoding. Taken together, these behavioral data further establish *C. elegans* as a valid model in which to study ethanol-induced impairments in long-term memory formation.

Although long-term memory was significantly affected by ethanol exposure during training, the effects of ethanol exposure on short-term memory for habituation were mixed. Ethanol did not affect short-term memory for habituation to stimuli presented at a short ISI (10 s) but there was mild alteration in habituation to stimuli presented at a long ISI (60 s; **Figure 2B**). It important to note that despite the modest change in habituation at a long ISI reported, ethanol-exposed worms still showed a significant decrement in response over time that reached an asymptotic response level that was significantly lower than naïve animals. In addition, although both 0.2 M and 0.4 M ethanol concentrations altered short-term habituation for stimuli presented at a long ISI to a similar degree, only training at 0.4 M blocked the formation of long-term memory. Thus, the effects of ethanol on long-term memory were unlikely due to a decrease in short-term habituation during training, and the mechanisms by which alcohol blocks formation of long-term memory may be distinct from those involved in impairment of short-term habituation. We earlier reported that in glr-1 mutant worms we saw normal short-term habituation and impairment of long-term memory for habituation (Rose et al., 2003).

Previous research from our lab showed that the activation of GLR-1 glutamate receptors during training is required for the behavioral expression of long-term memory for habituation that is correlated with a significant decrease in the punctal expression of GLR-1::GFP 24 h after training (Rose et al., 2003). In the current study, we found that ethanol exposure during training blocked both long-term memory for habituation as well as the decrease in GLR-1 subunit punctal expression. These results parallel previous studies that have shown experimental manipulations that block the training-induced down-regulation of GLR-1::GFP (i.e., protein synthesis inhibition between training blocks or after cue presentation) similarly block the behavioral indicators of long-term memory (Rose et al., 2003; Rose and Rankin, 2006). We hypothesized that the presence of ethanol somehow inhibited the training-induced decrease in GLR-1.

The unexpected increase in punctal GLR-1::GFP expression in the ethanol-exposed untrained condition (**Figure 3B**) suggested that ethanol alone increases GLR-1 expression and could potentially compete with, or counteract, any training-induced decrease in GLR-1. At a systems level, ethanol inhibits nervous system activity and as a compensatory mechanism, excitatory glutamatergic neurotransmission may be upregulated (Carpenter-Hyland et al., 2004; McCool et al., 2010; Zorumski et al., 2014). Ary et al. (2012) showed that ethanol increased GluR1 AMPA receptor subunit expression in the rodent nucleus accumbens. Work in mammalian cortical cultures found that ethanol increased expression of AMPA and NMDA receptor subunits (e.g., Hu et al., 1996; Chandler et al., 1999; Chandler, 2003), and increased GluR1 AMPA receptor subunits in the dopaminergic ventral tegmental area, specifically (Ortiz et al., 1995; Carlezon and Nestler, 2002). Thus, consistent with research in other model systems, the effects of ethanol on glutamate signaling may be the means by which ethanol affects memory.

The finding that ethanol increased the baseline level of GLR-1::GFP and impaired the training-mediated decrease in GLR-1::GFP led us to investigate the *nlg-1* gene as potentially important in mediating the effects of ethanol on memory in *C. elegans*. Neuroligins are a family of postsynaptic cell adhesion proteins that link to other synaptic proteins, including postsynaptic transmitter receptors (see Bemben et al., 2015 for review). Neuroligins are known to function in synaptogenesis (Scheiffele et al., 2000; Graf et al., 2004), synapse maturation and differentiation (Song et al., 1999; Levinson et al., 2005; Heine et al., 2008), and some forms of plasticity (Futai et al., 2007; Kim et al., 2008; Shipman and Nicoll, 2012). Because functional NLG-1 seemed to be necessary for the ethanol-induced increase in GLR-1::GFP, we tested whether *nlg-1* mutants would show a return of long-term memory and decreased GLR-1::GFP receptor expression despite ethanol exposure during training. When tested 24 h after training with ethanol, *nlg-1* mutant worms did form long-term memory for habituation (**Figure 4C**) and the memory-associated decrease in GLR-1::GFP punctal expression (**Figure 4E**). Memory was not seen in a transgenic NLG-1 rescue strain that expressed NLG-1 with its endogenous promoter.

Thus, functional neuroligin plays a role in alcohol-mediated impairment of memory mechanisms.

Previous mammalian studies suggest that neuroligin is required to preserve neuronal activity-dependent changes in AMPA receptor expression levels (Zeidan and Ziv, 2012). In *C. elegans*, the single *nlg-1* gene encodes orthologs of vertebrate neuroligin isoforms (Hunter et al., 2010) and *nlg-1* mutant phenotypes in *C. elegans* can be rescued by either human or rat neuroligin orthologs (Calahorro and Ruiz-Rubio, 2012), demonstrating that worm neuroligin has high functional homology with mammalian neuroligins. When we tested the effects of ethanol on GLR-1::GFP punctal expression in *nlg-1* mutant worms we found no difference in expression levels (**Figures 4E**, **5B**), thus confirming that functional NLG-1 protein plays an important role in the ethanol-induced increase in GLR-1::GFP. One possibility may be disruption of neuroligin-neurexin neuron-glial connections thus interfering with decreased glutamate transmission and neuron excitability by glial glutamate transporter GLT-1 (Aida et al., 2015; Katz et al., 2019; Walker et al., 2020). Alternatively, given the known roles of neuroligin (Südhof, 2008) it is possible that in *C. elegans* ethanol acts through some as yet unknown mechanism to increase *glr-1* expression levels, which perhaps require NLG-1 for stabilization. More research is needed to determine the exact mechanism by which ethanol and NLG-1 increase GLR-1::GFP expression levels.

The current study provides evidence that GLR-1 expression is increased by ethanol exposure, an effect that required the cell adhesion protein NLG-1. There is some evidence from mammalian neuron culture studies that suggest Nlgn1 mutations can reduce long-term potentiation (LTP; cellular correlate of memory; Shipman and Nicoll, 2012; Jedlicka et al., 2015); however, evidence is lacking with regards to neuroligins recruiting new AMPA receptors to synapses (a hallmark feature of LTP; Shipman and Nicoll, 2012; Bemben et al., 2015). Ethanol has been shown to either inhibit or reverse LTP (Izumi et al., 2005; Yin et al., 2007; Mishra et al., 2012; Avchalumov and Mandyam, 2020). Thus, Nlgn1 function may oppose the effects of ethanol with regards to LTP. It is difficult to test the role of NLG-1 in a glr-1 mutant in *C. elegans* as long-term memory for habituation is glutamate-dependent and completely absent in glr-1 mutant worms (Rose et al., 2003). Additional studies are needed to characterize the role of NLG-1 in ethanol-induced alterations in glutamate signaling.

Researchers that use simple model systems have identified shared sites of action for ethanol and have directly linked these findings to distinct behavioral effects. In *Drosophila*, Moore et al. (1998) identified a mutant with increased sensitivity to ethanol; this mutation disrupted the amnesiac gene, a gene originally identified in a screen for learning and memory deficits. From the current study it appears that, although habituation was modestly impacted by ethanol, the more significant deficit was seen with long-term memory. Our evidence suggests that memory mechanisms are perhaps not directly targeted by ethanol *per se*, but rather, that ethanol-induced memory impairment may result from disrupting or co-opting memory mechanisms (i.e., decreased GLR-1 expression in the case of habituation) perhaps to compensate for the depressive effects of ethanol on the nervous system.

The finding that neuroligin is involved in the effects of ethanol on AMPA receptor trafficking in several species reinforces the efficacy of how model systems can be useful to identify pathways affected by ethanol. Recent research in *Drosophila* by Petruccelli et al. (2018) demonstrated that alcohol can affect associative memories for reward through the conserved Notch molecule, another postsynaptic cell adhesion protein. In zebrafish (*Danio rerio*), Bertoncello et al. (2019) reported that ethanol acutely impaired memory consolidation for inhibitory avoidance learning; this work will hopefully lead to identification of genes critical for this effect. Taken together these data indicate that the detrimental effects of alcohol on learning and memory are highly conserved and often affect orthologous genes critical for human brain development and function.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JR composed multiple sections of the manuscript and revised figures. MB performed the initial long-term habituation study, confocal imaging and composed the original manuscript draft. JL and MP conducted additional confocal imaging trials and revised the manuscript. CL performed the qPCR experiments and revised the manuscript. CR conceptualized the study, consulted, supervised data collection, analysis throughout, composed, and revised all the manuscript drafts. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.908630/full#supplementary-material

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REVIEW
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The Brilliance of the Zebrafish Model: Perception on Behavior and Alzheimer's Disease

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Alzheimer's disease (AD) has become increasingly prevalent in the elderly population across the world. It's pathophysiological markers such as overproduction along with the accumulation of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT) are posing a serious challenge to novel drug development processes. A model which simulates the human neurodegenerative mechanism will be beneficial for rapid screening of potential drug candidates. Due to the comparable neurological network with humans, zebrafish has emerged as a promising AD model. This model has been thoroughly validated through research in aspects of neuronal pathways analogous to the human brain. The cholinergic, glutamatergic, and GABAergic pathways, which play a role in the manifested behavior of the zebrafish, are well defined. There are several behavioral models in both adult zebrafish and larvae to establish various aspects of cognitive impairment including spatial memory, associative memory, anxiety, and other such features that are manifested in AD. The zebrafish model eliminates the shortcomings of previously recognized mammalian models, in terms of expense, extensive assessment durations, and the complexity of imaging the brain to test the efficacy of therapeutic interventions. This review highlights the various models that analyze the changes in the normal behavioral patterns of the zebrafish when exposed to AD inducing agents. The mechanistic pathway adopted by drugs and novel therapeutic strategies can be explored *via* these behavioral models and their efficacy to slow the progression of AD can be evaluated.

Keywords: zebrafish, Alzheimer's disease, behavior, glutamatergic, cholinergic

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INTRODUCTION

Globally there has been a rise in the occurrence of neurological disorders such as Alzheimer's disease (AD), an advancing multifaceted neurodegenerative disorder, is the leading cause for 70% of dementia worldwide (Mayeux and Stern, 2012). The number of patients suffering from AD is predicted to reach 78 million globally by 2030 and expected rise to 139 million by 2050

(World Health Organization, 2021). The two main pathophysiological characteristic features of AD are formation of amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFT) (Sajjad et al., 2018; Hampel et al., 2021). Extracellular deposits of amyloid plaques form in the brain parenchyma and cerebral blood vessels, a condition termed as congophilic angiopathy or cerebral amyloid angiopathy (CAA)(Thanvi and Robinson, 2006; Jäkel et al., 2021). NFT are intracellular, large paired helical filaments of hyperphosphorylated tau proteins which cause synaptic and neural loss (Moloney et al., 2021). The predominant regions of the human brain which display AD pathology are the association areas of the cerebral cortex and the hippocampus (Wang et al., 2010). The vulnerable neurons among these regions are in the layer II of the entorhinal cortex, the subiculum and the CA1 region of the hippocampus since they are susceptible to display high levels of NFT and are first to be lost in early phases of the disease (Hyman et al., 1984; Kordower et al., 2001; Price et al., 2001). AD associated neuronal loss is observed, at specific cortical and subcortical brain sites such as the *trans*-entorhinal and entorhinal regions, hippocampus, amygdala, medial septal nucleus, nuclei of the diagonal band of Broca, basal nucleus of Meynert, compact part of the substantia nigra, locus coeruleus, midbrain, and pontine raphe nuclei. The neuronal loss is predominantly due to intra-neuronal deposits and extra-neuronal deposits of abnormal protein, which constitute the irregularly phosphorylated tau protein and the insoluble beta amyloid protein, respectively (Arnold et al., 1991; Stratmann et al., 2016). Further, dysregulation of neurochemicals such as acetylcholine (Ach), dopamine, glutamate, gamma amino butyric acid (GABA), serotonin and noradrenaline, due to neuronal loss in these critical regions has been observed in AD (Kaur et al., 2019). The manifested behavior in AD patients depend on the nature of development of the disease and progressively includes memory lapses, difficulty in organizing and planning of tasks, confusion, disorientation, changes in sleep patterns, anxiety, hallucinations, delusions, paranoia and lack of physical control (Mega et al., 1996; Serda, 2013). The neuropsychiatric symptoms (NPS) are based on disruptions in frontal-subcortical circuits (involving frontal cortex, basal ganglia, thalamus), cortico-cortical network (with hippocampus and amygdala as the epicenters), and the monoaminergic system [comprising of the neuronal cell bodies producing serotonin, norepinephrine, or dopamine located primarily in the brain stem (midbrain, pons, and medulla) and diffusely projected *via* long axons to virtually all parts of the brain to mediate human behavior] (Geda et al., 2013). These three neurobiological models form the foundations of neuroimaging and biomarker research in AD. It can be characterized into two main types, familial AD (FAD) and sporadic AD (SAD) (Piaceri et al., 2013).

Previously, AD animal models were developed to reproduce the brain lesions formed due to Aβ plaques and NFTs with the assumption that the models would authentically reproduce the human clinical condition. However, many therapeutics which have shown promise in the animal models have failed to show efficacy in clinical trials (Franco and Cedazo-Minguez, 2014). The challenges to translatability of the experiments include lack of good predictable animal models, lack of good biomarkers to indicate disease progression and diversity of genetic susceptibility of the target population under clinical trials (Franco and Cedazo-Minguez, 2014). The drawbacks of AD animal models such as rodents (including transgenics), canines, non-human primates for translation research are well discussed by Vitek and colleagues (Vitek et al., 2020) and will not be reiterated here. Emphasis, is on the development of current AD models that accelerate the translatability of the therapeutic interventions to check the progression of the disease (Götz et al., 2018; Li et al., 2019).

Zebrafish (*Danio rerio)* which belongs to the infraclass of teleost fishes is found to have certain features that makes it a suitable model for the study of AD and evaluation of therapeutic agents. Zebrafish possesses the following features; genes orthologous to the genes known to be involved in AD (Newman et al., 2011), a neuroanatomic alignment like humans (Guo, 2009; Kozol et al., 2016; Gupta et al., 2018) comparable neural signaling (Horzmann and Freeman, 2016) and the propensity of adult zebrafish and larval zebrafish to serve as models to study behavior (Norton and Bally-Cuif, 2010; Fero et al., 2011). These similarities make zebrafish an exceptional model for studying various neurodegenerative diseases comparable to *in vivo* and *in vitro* mammalian models for therapeutic drug screening (Michael Stewart and Kalueff, 2012). In recent years, the zebrafish model has proved to be useful in the study of neurodegeneration due to AD (Thawkar and Kaur, 2021). This review highlights the behavioral model development in both adults and larval zebrafish which enable the comprehension of AD progression mechanisms and efficacy of therapeutic interventions.

ZEBRAFISH AS AN ALZHEIMER'S DISEASE MODEL

Zebrafish has several characteristics that make it a suitable model for drug discovery. The zebrafish female can produce 200–250 eggs per mating, the embryogenesis is rapid i.e., the entire body structure is made 24 h post fertilization (hpf) and the internal organs like heart, liver and kidney develop 96 hpf. The development of the zebrafish can be visualized *in vivo* due to the transparency of the larvae. These fishes can be genetically manipulated and the gene expressions can be studied using adequate photo-imaging tools. Further, zebrafish embryos can be used to screen potential compounds in 50 microliter (μl) volumes (Chakraborty et al., 2009) (similar to an *in vitro* cellular model). Compounds added to water containing zebrafish, undergo the processes of absorption, distribution, metabolism and excretion similar to an *in vivo* model (Bhusnure et al., 2015). Zebrafish being a vertebrate, is evolutionary closer to humans than that of drosophila or the nematode models which are popular in neuroscientific and genetic studies (Shams et al., 2018). Using zebrafish in research, in lieu of animal studies involving mammals, is advantageous since it fulfils the principle of 3R's; replacement, refinement and reduction. The European Commission Directive in 2010, exempts the studies with larval

zebrafish up to 5 days post fertilization (dpf) from regulatory protection (Cassar et al., 2020). Therefore, studies with zebrafish larvae up to 5 dpf can be carried out as an alternative to testing in higher animals.

Thus, zebrafish applications in neurodegenerative disease research and neuropharmacology are greatly expanding due to lower economic costs, the small size of the organism, a sequenced characterized genome, and well described anatomical structures (Basnet et al., 2019). The most advantageous characteristics of zebrafish as a model would be the optical clarity of the embryo which would allow phenotypic visualization of the effects of genes all through the developmental process and the ease of introducing transient genetic manipulations and chemical manipulations (Koster and Sassen, 2015). All of these characteristics coupled with the presence of several orthologous genes in humans make zebrafish an ideal model to study AD as compared to rodents (Saleem and Kannan, 2018).

Neuroanatomical Comparisons Between Zebrafish and Mammalian Brain

Cognition includes all the mental processing by which knowledge is acquired, retained and used in perception, learning, memory and thinking. Despite not having a defined cortex or hippocampus like the mammalian brain, zebrafishes can perform required learning and memory tasks *via* various parts in brain that are functionally equivalent to these structures (Calvo and Schluessel, 2021; **Figure 1** and **Table 1**.

From a structural perspective, the zebrafish brain is similarly aligned as the mammalian brain possessing a forebrain (anterior most part), midbrain, hind brain (posterior most part) and a spinal cord (Guo, 2009). These develop at 24 hpf in the zebrafish. The forebrain of the zebrafish embryo further forms the telencephalon (composed by the pallium, the sub-pallium and the olfactory bulbs), the diencephalon, the hypothalamus and the retina (Vaz et al., 2019). The sub-pallium is the ventral telencephalon while the pallium is the dorsal telencephalon. The ventral telencephalon is sub-divided into two brain nuclei; the ventral nucleus (Vv) and the dorsal nucleus (Vd) of the ventral telencephalon. The dorsal telencephalon is much more complex and is composed of different brain nuclei or regions; the central zone (Dc), the dorsomedial zone (Dm), the lateral zone (Dl) and the posterior zone (Dp) of the dorsal telencephalon (Wullimann et al., 1996; Diotel et al., 2020). The zebrafish brain develops by eversion rather than inversion, due to which some classical regions of the mammalian brain, such as the hippocampus, amygdala, and substantia nigra are not present but the fish brain has structures which can carry out similar functions (Schmidt et al., 2013). Among the estimated 16 neurogenic niches of the zebrafish telencephalon, the Vv of the subpallium is comparable to the sub-ventricular zone (SVZ) of the lateral ventricle of mammals and the Dl, Dp are thought to be equivalent to sub-granular zone (SGZ) between the dentate gyrus (DG) and hilum of the mammalian hippocampus (Diotel et al., 2020; Ghaddar et al., 2021). The lateral pallium (LP) is predicted to be important for spatial learning, whereas the medial pallium (MP) is integral for avoidance learning in the zebrafish (Calvo and Schluessel, 2021). The diencephalon comprises of the thalamus, pineal body, and habenula (Vaz et al., 2019). The forebrain is responsible for receiving and processing sensory information and directing behavior. Telencephalon regulates social behavior, memory and emotion (Broglio et al., 2011) while the diencephalon addresses attention, alertness and circadian actions. Though the structures of telencephalon and diencephalon of the teleost brain are not seen in mammalian brain, some regions of the zebrafish and mammalian forebrain are conserved with respect to architecture and function (Vaz et al., 2019).

The midbrain (mesencephalon) of the zebrafish is important for vision and hearing (Vaz et al., 2019). It lies between the forebrain and hindbrain, containing the optic tectum, torus semicircularis, torus longitudinalis and the midbrain tegmentum (Zebrafish UCL, 2022). The region in mammalian brain functionally similar to the optic tectum, which has a role in vision in zebrafish, is known as the superior colliculus. The inferior colliculus of mammalian brain can be considered to be comparable to the torus longitudinalis which is responsible for auditory sensations in the zebrafish (Wullimann et al., 1996).

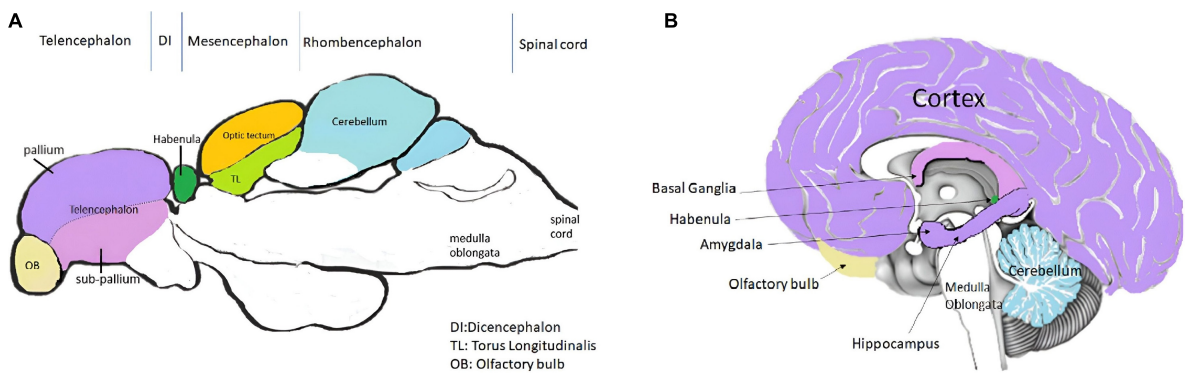

FIGURE 1 | Comparison of panel **(A)** (a representative of the mid-sagittal section of zebrafish brain) with **(B)** (a representative of mid-sagittal section of human brain). The figure attempts to represent the important areas involved in learning and memory in humans and zebrafish (Bally-Cuif and Vernier, 2010; Kozol et al., 2016; Langova et al., 2020).

TABLE 1 | Comparison of regions of mammalian brains with their homologous counterpart in zebrafish.

Sr. No	Mammalian brain	Homologous regions in zebrafish brain	Function in zebrafish	References
1.	Iso-cortex and the transitional cortex	Dorsal pallium	Control of short- term memory processes	Vargas et al., 2009
2.	Basal ganglia	Sub-pallium	Cognitive functions essential adaptive behavior such as planning, attention, learning and behavior	Cheng et al., 2014; Medina et al., 2014
3.	Hippocampus and amygdala	Lateral pallium and medial pallium	Control of sensory, motor and cognitive functions, like memory, learning and emotion	Ganz et al., 2015
4.	Habenula	Habenula	Control of motor and cognitive behaviors	Amo et al., 2010; Bühler and Carl, 2021
5.	Superior colliculus	Optic tectum	Control of vision	Wullimann et al., 1996
6.	Inferior colliculus	Torus longitudinalis	Control of hearing	Wullimann et al., 1996
7.	Medulla oblongata	Medulla oblongata	Control of respiration, circulation and wakefulness	Moens and Prince, 2002
8.	Cerebellum	Cerebellum	Control of motor reflexes, emotional learning and spatial cognition	Rodríguez et al., 2021

The hindbrain (rhombencephalon) of the zebrafish is composed of the posteriorly located medulla oblongata, the ventro-anterior pons and the dorso-anterior cerebellum (Moens and Prince, 2002). The cerebellum of the zebrafish is sub-divided into three parts; the vestibulolateralis lobe, the corpus cerebelli and the valvula cerebelli (Wullimann et al., 1996). The cerebellum controls motor reflexes and plays a role in emotional learning and spatial cognition in teleost fishes (Rodríguez et al., 2005). The fourth ventricle of the brain is formed by the medulla and pons which clubbed with the mid brain are referred to as the "brain stem." The brain stem regulates respiratory, circulatory and wakefulness activities in the teleost (Moens and Prince, 2002).

Functionally analogous regions in the brains of zebrafish and humans are illustrated and outlined in **Figure 1** (Bally-Cuif and Vernier, 2010; Kozol et al., 2016; Langova et al., 2020).

Neurotransmitter Pathways in Mammals and the Zebrafish

AD in humans is caused by excessive neuronal loss predominantly in the hippocampus and the cerebral cortex resulting into cognitive dysfunction, memory loss and behavioral changes in the patient. The symptoms of this disease are exacerbated when there is a massive loss of cholinergic neurons that synthesize Ach, a neurotransmitter involved in memory consolidation (Kaur et al., 2019). The currently approved therapy for AD includes acetylcholinesterase inhibitors (rivastigmine, galantamine, and donepezil), and a N-methyl D-aspartate receptor antagonist (NMDA) (e.g. memantine), and these are thought to preserve cholinergic neurotransmission (Guzior et al., 2014; Kaur et al., 2019) Besides ACh (Tohgi et al., 1994) several other neurotransmitters such as gamma amino butyric acid (GABA) (Zimmer et al., 1984), glutamate (Kuiper et al., 2000), serotonin (Volicer, 1985), dopamine (Pinessi et al., 1987) are present in reduced levels in the cerebrospinal fluid (Kaur et al., 2019) of AD patients and may contribute to the pathology of the disease.

Some AD patients exhibit extrapyramidal symptoms suggesting a loss of dopaminergic neurons (Lopez et al., 1997; Martorana and Koch, 2014). Aβ plaques, NFT, neuronal loss, and a reduction in dopamine content were observed in the neurons constituting the nigrostriatal Pathway. Thus, indicating that dopamine is clearly involved in the pathogenesis of AD. The zebrafish central nervous system uses similar neurotransmitters as the mammalian brain (GABA, glutamate, dopamine, noradrenaline, serotonin, histamine, and ACh) in both interneuron systems and in nerve pathways (Panula et al., 2006, 2010).

The differences and similarities in the metabolic and synthetic routes of these neurotransmitters in zebrafish and mammals are well reviewed by Horzmann and Freeman (2016) and Wasel and Freeman (2020). Three well researched neurotransmitter pathways i.e., cholinergic, glutamatergic, and GABAergic implicated in AD in zebrafish and humans (Santana et al., 2012) are described briefly.

Cholinergic Pathway

Ach is a significant neurotransmitter that is quite widespread in the human central nervous system (CNS) (Bierer et al., 1995). It is essential in learning processes and functions of memory and also helps in regulating the release of other neurotransmitters such as GABA. AD symptoms are manifested after a loss in cholinergic transmission in hippocampal region and the basal areas of the forebrain (Auld et al., 2002; Geula et al., 2021). The presence of acetylcholinesterase neurons in the telencephalon suggests the prevalence of a cholinergic system in the teleost. The telencephalon is a key area in the zebrafish brain for memory consolidation (Flood et al., 1976). The presence of cholinergic neurotransmission pathways has been validated in zebrafish by previous research as well (Williams and Messer, 2004; Mans et al., 2019). Studies using electrophysiological methods, histological methods, and antibody binding methods have indicated that the cholinergic system is widely distributed in the zebrafish brain (Mueller et al., 2004).

Glutamatergic Pathway

Glutamatergic pathway involving the NMDA receptor is one of the important excitatory pathways in vertebrates and is essential in learning, memory, and synaptic plasticity (Li and Tsien, 2009). However, increased amounts of glutamate in synaptic cleft can be neurotoxic (Maragakis and Rothstein, 2004).

Glutamate is a ligand to some classes of metabotropic receptors and ionotropic receptors. Human ionotropic glutamate

receptors (iGluRs) include, NMDA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors (Hu et al., 2012). Among ionotropic receptors, the NMDA receptor has been well characterized in zebrafish. Studies further proved that the telencephalon is sensitive to antagonists of NMDA receptors and that its long-term stimulation affects both memory and learning skills (Nam et al., 2004).

Reports on a family of excitatory amino acid transporters (EAATs) in humans, which transport the glutamate away from the synapse, have indicated that, neutralization of excess glutamate could reduce the excitotoxicity of the neuroreceptor (Danbolt, 2001). The presence of EAAT-orthologous genes in zebrafish makes it an ideal model to study this pathway (Rico et al., 2010).

GABAergic Pathway

GABA is an inhibitory neurotransmitter which regulates neural functions *via* modulating the activity of postsynaptic cells (Bowery and Smart, 2006). Defects in GABA neurotransmission plays a major role in CNS disorders (Staley et al., 1995). The GABAergic system is extensively present throughout the brain and plays a major role in balancing excitatory signals with inhibitory signals (Rissman et al., 2007). The equilibrium between the two is important in synchronization of various CNS functions. Through various pre-clinical and clinical studies it has been established that dysfunction of GABAergic system causes imbalance in excitatory and inhibitory signals. This imbalance is one of the potential markers for the initial stages of AD (Zheng et al., 2021). Neurodegenerative diseases can result from dysfunctional brain cells leading to changes in signaling systems (Lanctôt et al., 2004). The presence of GABAergic neurons and $GABA_A$ and $GABA_C$ receptors in zebrafish has been reported in the telencephalon, hypothalamus, tectum striatum, and olfactory bulb (Sadamitsu et al., 2021). Glutamic acid decarboxylase (GAD) is an enzyme that is conserved over species (Bosma et al., 1999). Reports of genes similar to human GAD genes in zebrafish were recorded and also their early stage expression was observed. GAD enzyme was majorly concentrated in the areas of the telencephalon medial longitudinal fasciculus in the midbrain, and at the border regions of the rhombomeres in the rostral hindbrain (Martin et al., 1998).

Administration of drugs and chemicals can modify the neurotransmission pathways by targeting various molecular mechanisms. Several compounds induce AD in mammals *via* one of these pathways (Cholinergic, Glutamatergic, and GABAergic). **Figure 2** elaborates the possible pathways by which certain inducers dysregulate signaling to promote AD.

GENES IMPLICATED IN HUMAN AND ZEBRAFISH ALZHEIMER'S DISEASE

Multiple studies have discovered that in FAD autosomal dominant mutations are mainly in three genes that are presenilin 1 (PS1), presenilin 2 (PS2), and Aβ precursor protein (APP) (Soto-Ospina et al., 2021). The expressed proteins of these genes are involved in ensuring functional cleavage of APP genes or in the formation of soluble Aβ protein. When mutated these

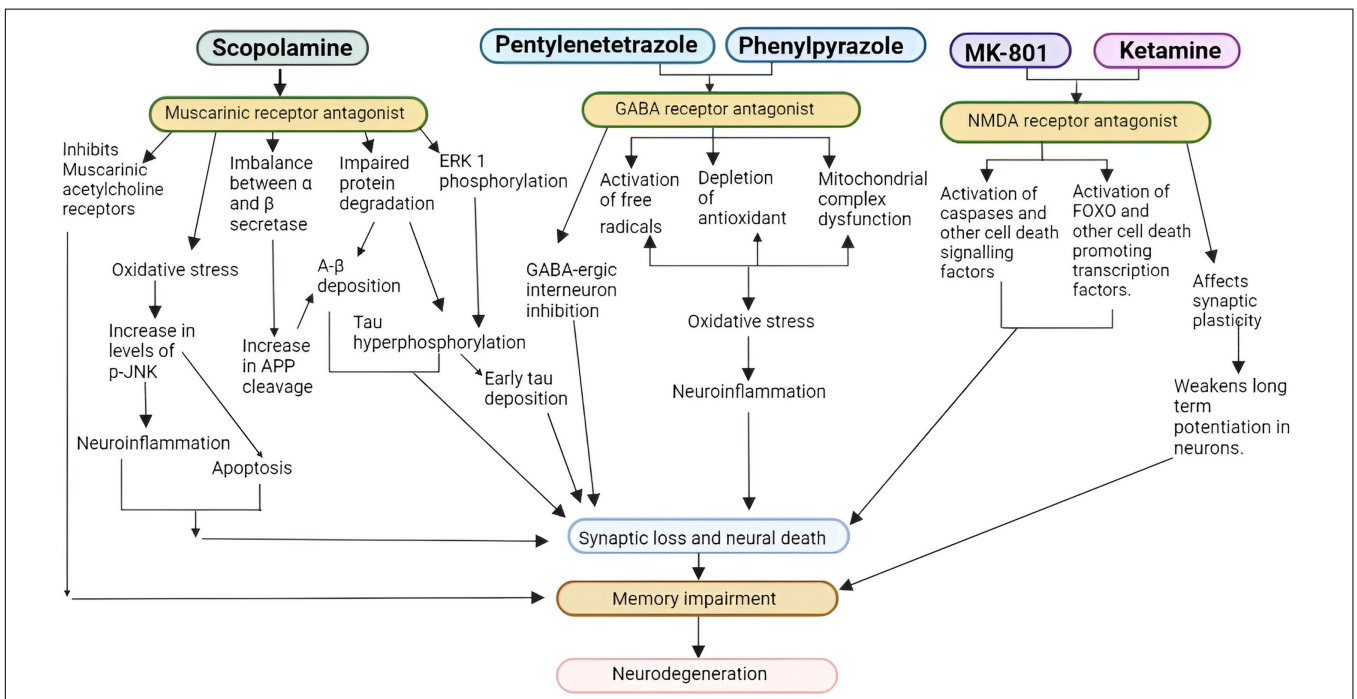

FIGURE 2 | Pathways for cholinergic, glutamatergic, and GABAergic inducers (FOXO, forkhead box transcription factor, p-JNK, p-Jun N-terminal kinase, APP, amyloid precursor protein, NMDA, N-methyl-D- aspartate, GABA, γ-amino butyric acid ERK1, Extracellular signal-regulated kinase 1) (Guan, 2008; Chen and Yeong, 2020).

proteins lead to the formation of insoluble Aβ which leads to Aβ plaque accumulation (O'Brien and Wong, 2011). SAD is correlated with the expression of the apolipoprotein E (ApoE) variant, ε4 (Xia, 2010). In zebrafish, various homologous gene encoding such as PS1, PS2, APP, and ApoE have been identified (Newman et al., 2014; Kiper and Freeman, 2021). **Table 2** enlists the genes that have a prominent role in AD and their orthologs in zebrafish.

BEHAVIORAL MODELS OF ALZHEIMER'S DISEASE IN ZEBRAFISH

Symptoms of AD in humans span from cognitive deficits (evidenced by decrease in memory, spatial recognition, problem solving, and language), abnormal motor movements (tremors, loss of co-ordination, incontinence, eating troubles) to behavioral and emotional issues (depression, agitation, anxiety, depression, tendency to hallucinate) (Voisin and Vellas, 2009; Blanc et al., 2014). The zebrafish when exposed to an AD inducing drug also manifest cognitive and memory impairments. The behavioral responses in the zebrafish can be grouped into basic motor responses which is inclusive of observed sensorimotor responses which in turn is encompassed by the higher learning and memory related reactions of the fish. The types of fish responses and the observed endpoints for behavioral assessments are well described in the publication by Tierney (2011). Major behavioral endpoints are conducted on the zebrafish 3–4 days post fertilization except learning or memory dependent endpoints (Tierney, 2011). The behavioral tests that can be performed on the zebrafish are shown in **Figure 3**. The AD inducing agents, effects of the moieties counter-acting AD in zebrafish and the behavioral model used for assessment are outlined in **Table 3**.

Models for Adult Zebrafish

Y-Maze Test

Over the last couple of decades, there has been a significant rise in interest in the behavioral features of zebrafish. However, the current protocols are based on reinforcement/reward or avoidance and have long training periods. The advantages of Y-maze are that, it is simple and has quick training sessions, allowing specific training of memory as it does not include

TABLE 2 | Orthologs of major human genes implicated in AD in the zebrafish.

Genes in humans	Function of gene	Gene Orthologs or co-orthologs in zebrafish	#Percentage identity of the expressed protein	References
PSEN1 *(Presenilin 1)* (Uniprot ID: P49768)	Catalytic subunit of gamma secretase complex that aids in the cleavage of APP	*psen1* (Uniprot ID: Q9W6T7)	72.4	Leimer et al., 1999; Fraering et al., 2004
PSEN2 *(Presenilin 2)* (Uniprot ID: P49810)	Catalytic subunit of gamma secretase complex that aids in the cleavage of APP	*psen2* (Uniprot ID: Q90ZE4)	71.3	Groth et al., 2002; Tu et al., 2006
APP *(Amyloid Precursor Protein)* (Uniprot ID: P05067)	Cell surface receptor that aids in neurite growth, neuronal adhesion and axonogenesis. The interaction of APP molecules on nearby cells promote synapse formation	*appa* (Uniprot ID: Q90W28)	72.65	Musa et al., 2001; Baumkotter et al., 2014
		appb (Uniprot ID: B0V0E5)	68.44	
MAPT *(Microtubule associated protein tau)* (Uniprot ID: P10636)	Promotes the stability and assembly of microtubules which help in establishing and maintaining neuronal polarity. It acts as a linker between C-terminal (binds axonal microtubules) and N-terminal (binds the plasma membrane components of the neuronal cells) of microtubules.	*mapta* *(Uniprot ID: Not assigned)* *maptb* *(Uniprot ID: Not assigned)*		Chen et al., 2009; Yoshida and Goedert, 2012; Sandberg et al., 2020
APOE *(Apolipoprotein E)* (Uniprot ID: P02649)	Plays a role in lipid homeostasis. It regulates lipid transport in the CNS which aids in neuron survival and sprouting.	*apoea* (Uniprot ID: Q503V2)	21.57	Fagan et al., 1996; Babin et al., 1997; Kowal et al., 1989; Sehayek and Eisenberg, 1991; Huang and Mahley, 2014; Kockx et al., 2018
		apoeb (Uniprot ID: O42364)	20.30	
(PSENEN) *(Presenilin Enhancer, Gamma-Secretase Subunit)* (Uniprot ID: Q9NZ42)	Essential subunit of gamma secretase complex that aids in the cleavage of APP	*psenen* (Uniprot ID: Q8JHF0)	77	Edbauer et al., 2003; Kimberly et al., 2003; Campbell et al., 2006; Zhou et al., 2019
BACE1 *(Beta Secretase 1)* (Uniprot ID: P56817)	Proteolytic cleavage of APP at the N-terminal between 671th and 672th residue which leads to the generation of beta cleaved soluble APP	*bace1* (Uniprot ID: A0A0G2KH37)	75.3	Hussain et al., 1999; Lin et al., 2000; Okada et al., 2010; Moussavi Nik et al., 2012
BACE2 *(Beta Secretase 2)* (Uniprot ID: Q9Y5Z0)	Proteolytic cleavage of APP between residues 690 and 691 also 671 and 672	*bace2* (Uniprot ID: Q5XJ89)	59.84	Esterházy et al., 2011; van Bebber et al., 2013
NCSTN *(Nicrastin)* (Uniprot ID: Q92542)	Essential subunit of gamma secretase complex that aids in the cleavage of APP	*Ncstn* (Uniprot ID: B3DGT7)	56.36	Yu et al., 2000; Edbauer et al., 2003; Lu et al., 2014; Bai et al., 2015; Lim et al., 2015; Zhou et al., 2019

[#]*Multiple alignment done by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and values obtained from the alignment scores.*

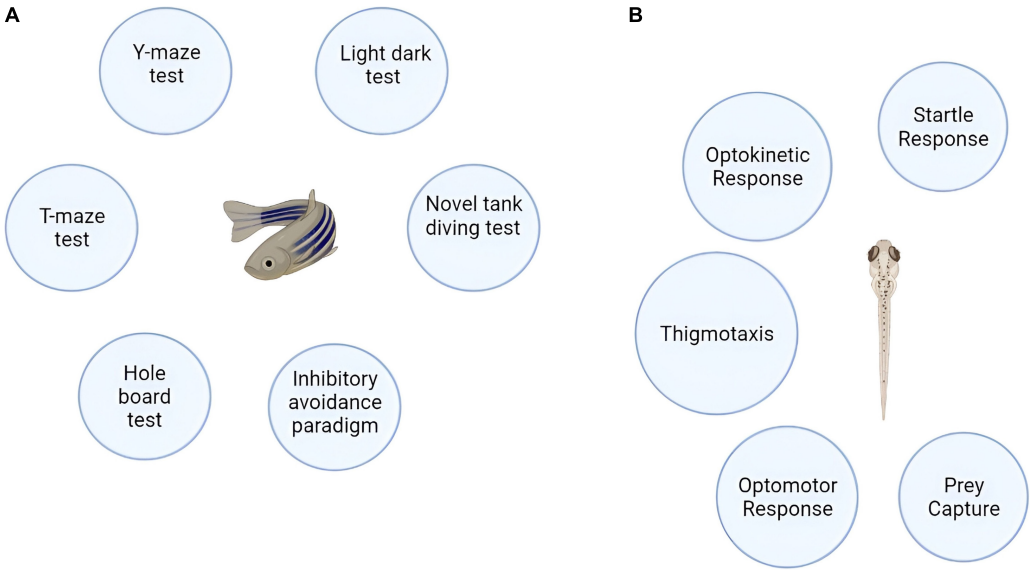

FIGURE 3 | (A) Behavioral models of adult zebrafish. **(B)** Behavioral models of zebrafish larvae.

conditioned learning. It also minimizes factors that might influence performance such as emotional and motivational states. This task assesses the natural tendency of the fish to explore a novel arm when the mnemonic demand is less. As the retention time does last for more than a couple of hours, the performance of the fish can be evaluated multiple times (Benvenutti et al., 2021). Advanced free movement pattern (FMP) Y-maze assay is a combination of existing aspects of the Y-maze assay with certain modifications in the analysis. In a study conducted by Cleal et al., the effects of periodic doses of D-Amphetamine and nicotine on cognitive behavior in zebrafish in the FMP-Y maze were observed for 14 days. The observed tetragram based on the alteration patterns were analyzed. It was observed that there was an improved memory function in the zebrafish in initial stages after a short withdrawal period along with a decrease in cognitive flexibility which was observed on D-amphetamine administration but not when treated with nicotine (Cleal et al., 2021).

The apparatus used for this test is a three-armed glass tank whose length, width, and height are 25, 8, and 15 cm, respectively. Each arm can be associated with different visual cues and it should be ensured that zebrafish is not biased toward a particular clue (Cognato et al., 2012). Two arms of the maze are opened during initial training and one arm remains blocked. It is observed that the novel arm once opened for exploration, zebrafish normally tend to spend more time in this arm (Zanandrea et al., 2018). This indicates that they are recognizing the novel arm and are willing to explore it. For disease-induced (*via* inducers such as scopolamine) zebrafishes, it has been observed that they have a lower intention of exploration and tend to float around in a particular arm. To create an unbias environment with regards to the cues the maze is rotated for each experiment (Valu et al., 2021b).

Spontaneous alterations analysis allows the measurement of spatial memory of the animals. The following is used to calculate the same:

$$\%\text{Alternation} = \text{Number of Alternations}/[\text{Total number of arm entries} - 2] \times 100$$

The higher the percentage of alteration the higher is the tendency to explore the novel arm which leads to an inference of low anxiety and good spatial memory (Kraeuter et al., 2019).

Hole Board Test

Hole board is a method normally used for the screening of prospective anxiolytic drugs. It is based on the hypothesis that the anxiety state of an animal is inversely proportional to its intention to look for baited holes. Initially, the animals are allowed to explore the experimental tank for 15 mins without any external visual cues in the open field and baited holes in the hole board. After habituation for a period of 4 days, training of zebrafish is commenced where only one hole of the whole board is baited. The time required to find the hole with the bait is noted and to avoid fixed directional swimming the fish is released into the tank from various locations. The maximum time given to each fish to find the baited hole is 3 mins (van der Staay et al., 2012; Bailey et al., 2015; Ruhl et al., 2015). This experiment tests the spatial cognition of the fish and therefore is an important model for testing of AD novel drug therapies.

T-Maze Test

T-maze is a method of assessing spatial memory, associative memory and learning in rodents and fishes. It has also been used in the evaluation of the pharmacological results of drugs in animal behavioral models. In zebrafish, this model is based

TABLE 3 | Table for zebrafish pathways, models, and drugs that affect those pathways.

AD Inducing agents	Age of zebrafish	Drugs against AD inducing agent	Molecular mechanism of the anti-AD drug	Behavioral tests conducted	References
Scopolamine	Adult 6 months	Li_2CO_3 (100 mg/L, 7 days)	Decrease p tau	Novel tank and Y-maze	Zanandrea et al., 2018
	Adult 3–4 months	*Thymus vulgaris* L essential oil (25–300 uL/L, 14 days)	Reduction in AChE activity and brain antioxidant capacity	Novel tank test, novel object recognition and Y-maze	Capatina et al., 2020
	Adult 6–8 months	Cotinine/6-hydroxy-L-nicotine (1–2 mg/L, 10 days)	Reduces oxidative stress and AChE activity and upregulates neuroprotective genes.	Novel tank diving, Y-maze and Object discrimination	Boiangiu et al., 2021
	Adult	Physostigmine (20 uM, 48 h)	AChE inhibitor and anxiolytic effect.	Passive avoidance	Kim et al., 2010
	Adult	*Streblus asper* 200–800 mg/kg 7 days	AChE inhibitor	Color-Biased Appetite Conditioning T-Maze and inhibitory avoidance	Singsai et al., 2021
	Larvae 168 hpf	*Convolvulus pluricaulis* 0.38 mg/ml 1 h	AChE inhibitor		Karunakaran et al., 2022
	Larvae 3dpf	Apigenin-rivastigmine hybrids 12.5 μg/mL	Antioxidant property, inhibits Aβ aggregation and exhibits anti-inflammatory property	Y-maze	Sang et al., 2020
	Adult 3-4 months	Hydroethanolic Extract of *Lycopodium selago* L. 3 mg/L 8 days	Inhibits AChE and has antioxidant properties	Y-maze, Novel tank and Novel object recognition	Valu et al., 2021a
	Adult 6–8 months	*Glycyrrhiza glabra* extract (250 mg/L) 30 mins	Cognitive improvement	T-maze and Novel object preference	Pusceddu et al., 2022
	Adult = 6 months	*Hericium erinaceus* ethanolic extract 3 mg/L 13 days	Enhances nerve growth factor (NGF) mRNA, increases protein expression in hippocampus along with antioxidant properties.	Y-maze, novel tank diving and novel object recognition	Valu et al., 2021b
	Adult < 8 months	Quercetin 20 ml/kg Single dose after 1 h exposure to scopolamine	Antioxidative property *via* free radical scavenging	Inhibitory avoidance and exploratory assessment	Richetti et al., 2011
	Adult <8 months	Rutin 20 ml/kg Single dose after 1 h exposure to scopolamine	Antioxidative property via free radical scavenging	Inhibitory avoidance and exploratory assessment	Richetti et al., 2011
	Adult 3–4 months	Agathisflavone 1–5 μg/L 8 days	Inhibits AChE activity and displays antioxidant activity	Novel tank diving and Y-maze	Dumitru et al., 2019
Aluminum chloride	Larvae 3 dpf	Linarin 16.7 μg/mL 3–5 dpf	Inhibits AChE activity	–	Pan et al., 2019
	Larvae 72 hpf	3-[4-(4-chloromethyl-benzoylamino)-phenyl]-8-methoxycoumarin 50 μg/mL 3 days	Inhibits AChE activity	Locomotor activity	Hu et al., 2019
	Larvae 2 dpf	Compound 4e 0.8 μg/mL 2–5 dpf	Inhibits $A\beta_{1-42}$ aggregation	–	Wang et al., 2021
	Larvae 2 dpf	TM-10 (Ferulic acid derivative) 0.33 μg/mL Single dose at 3 dpf	Inhibits $A\beta_{1-42}$ aggregation	–	Sang et al., 2019

(Continued)

TABLE 3 | (Continued)

AD Inducing agents	Age of zebrafish	Drugs against AD inducing agent	Molecular mechanism of the anti-AD drug	Behavioral tests conducted	References
	Larvae 3 dpf	*Cistanche tubulosa* (Schenk) wight 30 μg/ml 5 days	Inhibition of neuroinflammation by targeting TNF-α and IL-1β	–	Li et al., 2020
	Adult 6–8 months	Necrostatin-1 15 μmoL/L 30 days	Blocks necroptotic cell death by inhibiting receptor-interacting protein kinase-1 (RIP-1)	T-maze	Gao et al., 2022
	Adult 1 year old	Plasmalogen 3 mg/fish/day 8 weeks	Alleviating oxidative stress	Locomotor activty	Feng et al., 2021
	Juvenile 72 hpf	3-arylcoumarin Compound 2 (100 μg/ml) Compound 20 (50 μg/ml) Compound 22 (100 μg/ml)	Inhibition of monoamine oxidase B	Locomotor activity	Yang et al., 2019
	Larvae 3 dpf	Apigenin-rivastigmine hybrids 12.5 μg/mL	Antioxidant property, inhibits Aβ aggregation and exhibits anti-inflammatory property	Y-maze	Sang et al., 2020
Okadaic acid	Adult 12-15 months	4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5- dione 1μM 9 days	Normalizes PP2A activity, phosphorylated tau and inhibition of GSK3β	Learning and memory	Koehler et al., 2019
	Adult 12-15 months	Lanthionine ketimine-5-ethyl ester 500 μM 9 days	Increasing levels of Brain-derived neurotrophic factor, Protein kinase B and cAMP response element-binding protein. Decreases apoptosis.	Learning and memory	Koehler et al., 2018
Aβ injection	Larvae 1 dpf	LiCl 100 μM 1–5 dpf	Decrease of p-tau	Locomotor and bouncing ball avoidance	Nery et al., 2014
	Larvae 2 dpf	TM-10 (Ferulic acid derivative) 0.33 μg/mL Single dose at 3 dpf	Inhibits butyrylcholinesterase activity, monoamine oxidase activity and aggregation of Aβ	–	Sang et al., 2019
	Larvae 5 days	β casein coated-gold nanoparticles (βCas AuNPs) 3 ng Au per 4.5 ng βCas 3–5 days (at different time intervals)	Inhibits Aβ plaque formation and reactive oxygen species formation. Recovering synaptophysin.	Locomotor activity	Javed et al., 2019
	Adult and larvae (5 dpf)	LDC8 (pyrazolotriazine derivative) Single dose	Decreases phosphorylated tau formation and inhibits GSK3β and CDK-5 activity	–	Reinhardt et al., 2019
Pentylentetrazole	Larvae 7 dpf	6-gingerol 12.5–37.5 μM 24 h	It acts as an inhibitor of NMDA containing NR2B channel	Locomotor activity	Gawel et al., 2021

AChE, acetylcholine esterase; TNF α, tumor necrosis factor α; IL-1β, Interleukin-1β; PP2A, protein phosphatase 2A; GSK3β, glycogen synthase kinase 3β; cAMP, cyclic adenosine monophosphate; CDK-5, cyclin dependant kinase-5; NMDA, N-methyl D-aspartate.

on how quickly it can grasp a certain behavior through discrimination training which contains multiple sessions of training and contains a stimulus or food reward. The fish is trained to choose an arm through pavlovian conditioning (Gould, 2011; Bault et al., 2015).

The apparatus has one long arm which is the starting zone for the zebrafish and two short arms among which one is the favorable zone and the other one is the unfavorable zone These zones can also be color-coded. During training, the zebrafish is allowed to acclimatize within the two arms it can choose. If it chooses the wrong arm, it meets unfavorable conditions such as disturbance. Upon choosing the correct arm the zebrafish does not face any disturbance or can get a positive reinforcement such as food. On the day of acquisition, time spent in unfavorable and favorable zones is noted down. The control zebrafish will spend more time in the favorable zone and the AD model will not be able to develop such a bias. In the recent research conducted by Moreira et al., the effects of oxybenzone on behavior and cognition was tested by using various behavioral models including T-maze test. The results showed a decrease in the explorative instincts of the animal and impairment of the animal's memory (Moreira and Luchiari, 2022).

Inhibitory Avoidance Paradigm

The apparatus consists of an aquarium which is of the dimensions 60 cm × 30 cm × 30 cm and contains about 10 cm water level. This aquarium is to be divided into two compartments with the help of a manually operating sliding door and the surfaces of each compartment are white and black, respectively. The black compartment is wired to a power source of 1-9V (AC). After allowing the fishes to acclimatize in both the compartments, an electric shock is given in the dark compartment for about 5 s and then the fish is returned to its home tank (Manuel et al., 2014). The following day the trained fish's ability to avoid the dark compartment is observed. The fishes that do not enter the dark compartment in 3 mins are marked as successful in exercising their memory to avoid making the adverse choice and in the AD model, the fishes would show no such bias due to memory loss (Manuel et al., 2014).

Novel Tank Diving Test

For several animals, including zebrafish, new surroundings can be anxiogenic. This test has been considerably used in the modeling of anxiety and assessing the effects of anxiolytics. As this test is based on the idea of a novel surrounding, it is equivalent to the open field test generally performed in rodents. In this test, the initial response of an adult zebrafish is to stay at the bottom of the tank until they get accustomed to the rest of the tank (Stewart et al., 2012; Haghani et al., 2019).

In this method, the fishes are paired and transferred into a behavioral examination tank. After holding the fishes in the behavioral tanks, they are transferred into separate 100 ml beakers containing 25–30 mL water for immediate transportation into observational tanks. After the transfer, the fishes are observed in the new environment. The ability of the fishes to explore the novel tank is investigated. The more amount of time a fish spends at the bottom of the tank indicates high anxiety levels, which is a common manifestation in neurodegenerative disease models (Haghani et al., 2019).

Anwer et al. (2021) have recently hypothesized that this test when conducted in taller tanks yields a high repeatability.

Light Dark Test

The light-dark test is commonly used in the quantification of anxiety in rodents but in recent years has been also used for screening adult zebrafish. This model uses the nature of phototaxis displayed in fishes to evaluate the anxiety (Facciol et al., 2017). It is a straightforward and efficient method which does not require pre-training and enables understanding of pharmacological modifications in zebrafish. Further pharmacological studies observed that the method is sensitive to anxiolytic but not to the panicolytic drugs. In nature, a preference for the dark allows them to evade predators rather than being exposed in a white background. Anxiety inducing drugs alter zebrafish's tendency to explore the tank and they stay in the dark region of the tank for longer periods compared to control, displaying negligible or low explorative preferences. The light-dark apparatus that is used for rodents is modified accordingly for application in zebrafishes. The tank has dimensions of 15 cm height × 30 cm length × 16 cm width (Stewart et al., 2011). The major differences in the apparatus are that it is sealed to avoid leakage of water which is filled to a height of 12 cm, there is also no sliding door between the two sides (light and dark) hence the fish are free to swim in both the compartments (Stewart et al., 2011). Currently several inducers of neurological diseases are being investigated to test for manifestations of symptoms like anxiety using this model. Decynium-22 was tested by Maximino et al. for inducing anxiety using the light dark test model (Maximino, 2021).

Models for Larvae Zebrafish

Startle Response

Zebrafish larvae when exposed to abrupt stimuli of unexpected touch (tactile), loud sound (acoustic), or sudden bright light (visual) tend to react by a swimming burst to escape from the threatening stimuli (Fetcho and McLean, 2009). Startle response is important to understand whether the larvae have proper sensory and motor stimuli which are critical for survival (Troconis et al., 2017; Faria et al., 2019). The different times for development of different responses are listed in **Table 4**.

This behavioral model is important for neuropharmacological research to observe if the drugs being administered can restore the normal response to startling stimulus in larvae (Basnet et al., 2019; Banono and Esguerra, 2020). Locomotion analysis in larvae *via* high-throughput methods will be able to provide observations required for testing and identification of neuroactive compounds. Recent research by Png et al. (2021) used acoustic startle response with the help of 36 continuous mechanical taps, to observe the impairment of cognitive ability in response to 0.8 uM morphine administration for 5 days starting from 5 h post fertilization.

Optokinetic Response

The optokinetic response is first observed in zebrafish 73 h post-fertilization and it slowly develops until 4 days post fertilization where it reaches to 9/10th of an adult goldfish (Easter and Nicola, 1997). Around the time zebrafish larvae start hunting for food, the response is completely developed. There are various types of optokinetic responses but the horizontal optokinetic response is the only one studied in zebrafish and it is also commonly studied in other species. This response signifies a stereotyped eye movement in response to any form of movement within the field of vision (Huang and Neuhauss, 2008). To evoke an optokinetic response in zebrafish larvae an LCD screen displaying moving graphics or a rotating drum containing black and white stripes is used (Cameron et al., 2013).

Effect on the optokinetic response *via* chemicals that affect the central nervous system allows the study of Alzheimer's induction

TABLE 4 | Type of different startle responses and their time of development.

Type of startle response	time for development (days post-fertilization)	References
Tactile	2	Colwill and Creton, 2011
Visual	3	Ganzen et al., 2017; Tucker Edmister et al., 2022
Acoustic	5	Best et al., 2007

and treatment by considering this response as an analysis parameter (Barbereau et al., 2021). This behavioral model has been used to study the effect of drugs that reduce anxiety such as fluoxetine. It was observed that the anxiolytic drug expunged the consequences (Hodges, 2021).

Thigmotaxis

When exposed to a new environment animals tend to have a natural tendency of moving to the periphery of the novel environment. This behavior, also known as the wall-hugging behavior, is used to estimate anxiety and it is conserved across all species (Simon et al., 1994; Colwill and Creton, 2011). Zebrafish larva shows thigmotaxis in response to novel environments at 5 days post fertilization. Exposure to sudden darkness or sudden light can elucidate this behavior in zebrafish (Liu et al., 2016). The test should be performed in an arena where there is enough space to distinguish between inner and outer zones so the choice is usually a 24 well plate (Schnörr et al., 2012).

This test can be used to study anxiety-like behavior in the zebrafish (Cho et al., 2012). Thus, using this behavioral model one can study the induction of Alzheimer's like phenotype and efficacy of anxiolytic drugs (Ahmad and Richardson, 2013). Recent studies have been conducted to test the anxiolytic effect of compounds like Buspirone hydrochloride by observing thigmotaxic behavior in zebrafish as it was a responsive and straightforward way for evaluating the efficacy of similar anxiety reducing drugs (Abozaid and Gerlai, 2022).

Optomotor Response

Zebrafish can be exposed to aversive and non-aversive visual cues on an LCD screen monitor. For responsive cues, 15 zebrafish larvae are placed in a single petri dish and then placed on a monitor. The zebrafish is exposed to moving red and white stripes 5 days post-fertilization (Fleisch and Neuhauss, 2006; Creton, 2009; Nery et al., 2017). The directions of the stripes are alternated every 1 min with a 5-s interval, where the cue is faded (Karaduman et al., 2021). The animals usually tend to swim in a direction similar to the movement of the stripes. The petri plate is divided into three zones to observe the number of fishes present in the correct zone. In aversive cues, the LCD monitor displays a red bouncing ball. The animals will try to swim to the non-stimulated area of the petri dish to escape the bouncing ball (Pelkowski et al., 2011; Nery et al., 2014). These tests help in determining the cognitive performance of animals (Karaduman et al., 2021). Quinpirole, a compound that induced anxiety-like symptoms by targeting dopaminergic signaling, was observed to impair optomotor response (Nabinger et al., 2021).

Prey Capture

After hatching, zebrafish embryos start swimming toward the prey, a behavior that is essential for survival (Muto and Kawakami, 2013). To test this behavior a small air bubble released in the test arena is utilized as prey (Gahtan et al., 2005). In the assay, the latent period before the attack on the prey, the number of attacked or captured prey, and the efficiency with which the prey is captured are the parameters measured (Tierney, 2011). The convergence of eye movement marks the onset of hunting behavior and the prey attack is defined by the biting motion of larvae (Bianco et al., 2011). This behavior involves decision making therefore can be used to assess cognitive behavior (Formella et al., 2018).

CONCLUSION AND FUTURE PROSPECTS

The zebrafish model is a robust system for the physiological and genetic study of AD (Xia, 2010). The advantages of the zebrafish models are that they allow sizable forward genetic screening, investigation of different properties such as temporal, spatial, and also real-time observation of the pathological changes *in vivo*, exploration of behavioral and pharmaceutical in both larvae and adults (Lorent et al., 2004). Over the years, extensive research has provided abundant evidence and essential information about the correlation between disease and its development in zebrafish (Brun et al., 2021). Due to advancements in modern technology, the zebrafish system can be established as a substitute to mammalian models in drug development, target identification, and validation along with providing shorter routes for novel therapeutic strategies (Chakraborty et al., 2009). The larva-adult duality in the zebrafish model has a large-scale utilization for preclinical studies before rodent models for validating novel therapies (Bailey et al., 2015). The article sheds light on the use of behavioral models of both the larva and adult zebrafish along with the different neurotransmitter pathways that regulate them but strategies to overcome the complexities are yet to be established (Santana et al., 2012; Saleem and Kannan, 2018). Zebrafish as a model could assist in further understanding neuroanatomical circuits and their role in neurodegenerative disorders (Stewart et al., 2015). Further research in regard to zebrafish telencephalon is required to gain an understanding of the neurological and molecular mechanism at the onset of AD. This study would allow us to extrapolate the observations and corelate them to the early biomarkers of mammalian AD commencement in the hippocampus. This would assist in the progress of novel preventive therapies. It can be concluded that the zebrafish is a promising model for gaining a perception of mechanisms of AD but requires further optimization to be indispensable to novel drug screening.

AUTHOR CONTRIBUTIONS

GK and AU visualized the presented idea, contributed to the manuscript writing, supervised the project and corrected, revised, and approved the manuscript. MB, SB-P, and AS contributed equally to doing literature searches and in the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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Event-Related Potential Evidence for Involuntary Consciousness During Implicit Memory Retrieval

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Classical notion claims that a memory is implicit if has nothing to do with consciousness during the information retrieval from storage, or is otherwise explicit. Here, we demonstrate event-related potential evidence for involuntary consciousness during implicit memory retrieval. We designed a passive oddball paradigm for retrieval of implicit memory in which an auditory stream of Shepard tones with musical pitch interval contrasts were delivered to the subjects. These contrasts evoked a mismatch negativity response, which is an event-related potential and a neural marker of implicit memory, in the subjects with long-term musical training, but not in the subjects without. Notably, this response was followed by a salient P3 component which implies involvement of involuntary consciousness in the implicit memory retrieval. Finally, source analysis of the P3 revealed moving dipoles from the frontal lobe to the insula, a brain region closely related to conscious attention. Our study presents a case of involvement of involuntary consciousness in the implicit memory retrieval and suggests a potential challenge to the classical definition of implicit memory.

Keywords: implicit memory, consciousness, mismatch negativity, P3, pitch interval

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INTRODUCTION

Numerous studies have been carried out for investigation into memory and learning since the 19th century. Classical notion claims that memory is implicit (non-declarative) if has nothing to do with consciousness during the information retrieval from storage, or is otherwise explicit (declarative). Under this notion, the so-called implicit memory refers to nonconscious memory abilities such as musical skills (e.g., play a piano). It is an important type of memory which often has an automatic quality and such a quality for the purpose of surviving in this complex diverse world is often innate. For instance, birds rely on it to fly in the sky and aquatic animals rely on it to live in the water. A well-known case for manifesting implicit memory is the amnesic patient H. M. who had undergone temporal lobe resection. H. M. preserved the memory related to motor skills and perceptual learning (Corkin, 1968; Moscovitch, 1995) as well as the types of memory that depended on the brain areas other than the medial temporal lobe, particularly on the hippocampus. H. M. also preserved the memory that did not depend on conscious awareness (Schacter, 1987; Squire, 1992).

In addition, studies have revealed that selective injury to the medial temporal lobe leads to an isolated deficit in long-term memory (Scoville and Milner, 1957; Reber, 2013). The medial temporal lobe is equated with conscious-forms of memory and, therefore, with explicit memory (Degonda et al., 2005) which is commonly linked with the conscious awareness of memory retrieval.

Some studies show that an intact hippocampus is necessary for rapid associative learning with and without consciousness for long-term and short-term storage (Bennington and Polich, 1999; Henke, 2010). Imaging findings suggest that new semantic associations can be formed and retrieved by way of the medial temporal lobe without awareness of these associations (Henke et al., 2003a). The imaging studies further suggest that conceptual knowledge acquired during masking can be unconsciously retrieved (Henke et al., 2003b) and that implicit semantic associative learning engages the hippocampus and influences explicit memory (Degonda et al., 2005). Additionally, a visual electrophysiological study provides evidence for recognition lacking two hallmark explicit memory features: awareness of memory retrieval and facilitation by attentive encoding (Voss and Paller, 2009). All these studies suggest that consciousness seems to be a weak criterion for differentiating explicit and implicit memories. A new model has therefore been proposed in which memory systems are distinguished based on the processing characteristics involved rather than by the role of consciousness (Henke, 2010). The new model is an alternative to the classical memory model based on evidence from behavioral studies conducted in brain-impaired patients. To date, evidence for this new insight into distinguishing types of memory largely comes from subjective behavioral studies in brain lesion patients or neural imaging studies of explicit memory at spatial resolution. However, memory studies in healthy subjects at the temporal dimension during implicit memory retrieval is limited.

Mismatch negativity (MMN) is an auditory event-related potential (ERP) component and reflects the auditory cortical responses to novel stimuli (Näätänen et al., 1978, 2007; Partanen et al., 2013). MMN has been widely used as an effective neural marker for early auditory processing at a pre-attention stage (Luo et al., 2006; Gu et al., 2012; Wang et al., 2013; Guo et al., 2018). Importantly, MMN is regarded as a probe of implicit memory. P3 is another ERP component (Sutton et al., 1965) and has been claimed to be a neural marker of conscious perception by a number of investigators (Babiloni et al., 2006; Del Cul et al., 2007; Dehaene and Changeux, 2011; Rutiku et al., 2015). In the present study, we used MMN and P3 to investigate whether or not implicit memory is truly unassociated with consciousness. Specifically, we used an implicit memory paradigm (van Zuijen et al., 2006; Schröger, 2007) to expose a group of amateur musicians and a group of non-musicians to two different types of pitch intervals (e.g., one-pitch interval [C4 – C#4] and four-pitch interval [C4 – E4]). We found that a significant P3 component following the MMN was evoked in the amateur musicians, but was not in non-musicians. Our results provide ERP evidence that implicit memory retrieval of the musical pitch interval involves involuntary consciousness.

MATERIALS AND METHODS

Whole-Head Electroencephalogram Recording

Participants

Thirty-six healthy students with normal hearing and no history of neurological disorders or learning abnormalities from the University of Science and Technology of China (USTC) participated in the present study (20 males, mean age = 21.85 years, SD = 1.84, right-handed; 16 females, mean age = 21.19 years, SD = 2.48, right-handed). Participants were allocated into the amateur musician group (10 males, mean age = 21.20, SD = 1.32; 8 females, mean age = 19.63, SD = 2.06) and the non-musician group (10 males, mean age = 22.50, SD = 2.12; 8 females, mean age = 22.75, SD = 1.83) according to their experience of musical training. Amateur musicians were recruited from Student Symphony Orchestra, Student Chinese Orchestra, and Student Choir at USTC and they had musical training more than 10 years for playing violin, piano, flute, Chinese zither, pipa, or singing. Amateur musicians and non-musicians were age- and sex-matched. The experimental protocols and procedures were reviewed and approved by the Biomedical Research Ethics Committee of the University of Science and Technology of China.

Stimuli

Auditory stimuli used in the present study were Shepard tone pairs, which were synthesized with Praat software (Institute of Phonetic Sciences, University of Amsterdam, Netherlands[1]). The tone pairs were edited by Adobe Audition software. Each of the Shepard tones consists of many sinusoidal components locked at successive intervals of an octave simultaneously. In contrast to harmonic tones, which are well defined in terms of both pitch chroma and height, Shepard tones are well defined in terms of pitch class (C, C#, D, etc.) but poorly defined in terms of height, since the usual cues for height attribution are missing (Deutsch, 1986). The positions of the envelope within the lower octave peaked at C4 (262 Hz, f_{min} = 32.7 Hz), C#4 (277 Hz, f_{min} = 34.7 Hz), D4 (294 Hz, f_{min} = 36.8 Hz), D#4 (311 Hz, f_{min} = 39.0 Hz), E4 (330 Hz, f_{min} = 41.3 Hz), F4 (349 Hz, f_{min} = 43.8 Hz), F#4 (370 Hz, f_{min} = 46.4 Hz), G4 (392 Hz, f_{min} = 49.2 Hz), G#4 (415 Hz, f_{min} = 52.1 Hz), A4 (440 Hz, f_{min} = 55.2 Hz), A#4 (466 Hz, f_{min} = 58.5 Hz), and B4 (494 Hz, f_{min} = 62.0 Hz) (**Table 1**). Four types of Shepard tone pairs were presented: pairs with one-pitch and four-pitch clockwise intervals and one-pitch and four-pitch counterclockwise intervals (**Table 2**). Clockwise here means the second tone of each tone pair is always rising (ascending, **Figure 1B**), whereas counterclockwise means the second tone is always falling (descending, **Figure 1B**). The standard stimuli consisted of the one-pitch (clockwise) interval tone pairs, comprising C4 – C#4, C#4 – D4, D4 – D#4, D#4 – E4, F4 – F#4, G4 – G#4, A4 – A#4, and A#4 – B4, and the one-pitch (counterclockwise) interval tone pairs, including C#4 – C4, D4 – C#4, D#4 – D4, E4 – D#4, F#4 – F4, G#4 – G4, A#4 – A4, and B4 – A#4 (**Table 2**). The four-pitch

[1]www.praat.org

TABLE 1 | Minimum frequencies (in Hz) of Shepard tones.

Note name	Minimum Frequency (Hz)	Note name	Minimum Frequency (Hz)
C4	32.7	F#4	46.4
C#4	34.7	G4	49.2
D4	36.8	G#4	52.1
D#4	39.0	A4	55.2
E4	41.3	A#4	58.5
F4	43.8	B4	62.0

C: do; D: re; E: mi; F: fa; G: so; A: la; B: xi.

TABLE 2 | Two blocks of tone pairs with one-pitch and four-pitch interval.

Clockwise (block 1)		Counterclockwise (block 2)	
One-pitch interval	Four-pitch intervals	One-pitch interval	Four-pitch intervals
C4 – C#4	C4 – E4	C#4 – C4	E4 – C4
C#4 – D4	C#4 – F4	D4 – C#4	F4 – C#4
D4 – D#4	D4 – F#4	D#4 – D4	F#4 – D4
D#4 – E4	D#4 – G4	E4 – D#4	G4 – D#4
F4 – F#4	E4 – G#4	F#4 – F4	G#4 – E4
G4 – G#4	F4 – A4	G#4 – G4	A4 – F4
A4 – A#4	F#4 – A#4	A#4 – A4	A#4 – F#4
A#4 – B4	G4 – B4	B4 – A#4	B4 – G4

(clockwise) interval tone pairs consisted of C4 – E4, C#4 – F4, D4 – F#4, D#4 – G4, E4 – G#4, F4 – A4, F#4 – A#4, and G4 – B4, and for the four-pitch (counterclockwise) interval tone pairs, the tones were reversed in direction, that is, E4 – C4, F4 – C#4, F#4 – D4, G4 – D#4, G#4 – E4, A4 – F4, A#4 – F#4, and B4 – G4 (**Table 2**). The one-pitch and four-pitch interval Shepard tone pairs served as the standard and deviant stimuli, respectively. Each tone was 100 ms in length, and each tone pair was 500 ms in length. The within-pair interval was 300 ms, stimulus onset asynchrony (i.e., from the onset of one tone-pair onset to the next) was set to 1,600 ms (**Figure 1A**).

Procedure

Participants sat in a comfortable sofa in an electrically shielded soundproof room. During whole-head electroencephalogram (EEG) recording, the subjects were instructed to ignore the auditory stimuli and watch a muted movie of their choice with subtitles. The stimuli were diotically presented through headphones (Sennheiser HD 25) at an intensity of ∼70 dB sound pressure level. To rule out the possibility that the MMN evoked by the deviant tone pairs was simply a response to an infrequent note in the deviant stimuli, we constructed the deviant tone pairs from the tones that also appeared among the standard stimuli (**Table 2**). The standard stimuli were presented with a probability of 7/8, and the deviant was presented with a probability of 1/8. In block 1 (**Figure 1B**, *upper panel* and **Supplementary Audio 1**), the stimuli were all the clockwise variants; each one-pitch interval tone pair was used as a standard stimulus with a probability of 7/64, and each four-pitch interval tone pair was used as a deviant stimulus with a probability of 1/64. In block 2 (**Figure 1B**, *lower panel* and **Supplementary Audio 2**), the stimuli were all the counterclockwise variants; each one-pitch interval tone pair was used as a standard stimulus with a probability of 7/64, and each four-pitch interval tone pair was used as a deviant stimulus with a probability of 1/64. The blocks were presented separately twice for 15 min each. Both amateur musician and non-musician participated in both blocks.

Electroencephalogram Recording and Preprocessing

Whole-head EEG signals were recorded using a SynAmps RT amplifier (NeuroScan, Charlotte, NC, United States) with a cap carrying 64 Ag/AgCl electrodes placed on the scalp at specific locations according to the extended international 10–20 system. Data were recorded at a sampling rate of 500 Hz. The reference electrode was attached to the tip of the nose, and electrode AFz served as the ground during the recording. To minimize the artifacts induced by eye-movement, horizontal and vertical eye movements were recorded using two bipolar electrooculography (EOG) electrodes. All electrode impedances were maintained below 5 k**Ω**. Preprocessing and data analysis were performed with NeuroScan and SPM12.[2] Artifact rejection, filtering, and averaging were performed offline using Scan 4.3 (Neuroscan; Compumedics). The EEG data from the whole-head recordings were offline band pass (1–30 Hz) filtered with a finite impulse response filter. The filtered continuous data were then segmented into 900 ms epochs, including a 100 ms prestimulus baseline epoch. At the trial level, epochs with fluctuations in amplitude of at least 50 μV were considered artifacts and rejected expect for those of the EOG channels, which were excluded from the averaging. For illustration purposes, the ERPs of non-musicians and amateur musicians to the standard stimuli were averaged across the one-pitch interval tone-pair stimuli; a similar procedure was used for the responses to the deviant stimuli. The normality of the raw EEG data was assessed by using the Shapiro–Wilk (S-W) test. Paired-sample *t*-tests were performed at each sampling point throughout the epoch (−100 to 800 ms, one sampling point per 2 ms) for all subjects. Group-averaged deviant-minus-standard difference waveforms were then obtained by subtracting the ERPs evoked by the standard stimuli from those evoked by the deviant stimuli. The MMN component was then identified as a positive phase reversal over the mastoid processes (M1 and M2), and P3, was identified as the evoked signal immediately following the MMN component. Inspection of the grand-averaged difference wave suggested that the MMN peak amplitude was largest at FCz among the midline electrodes, which is consistent with findings in the literature indicating that the MMN component is prominent at the frontocentral sites (Näätänen et al., 2007). Two time windows were selected for amplitude measurements. Time window was 30-ms wide, ranging from 15 ms before the peak of the MMN recorded from electrode FCz to 15 ms after the peak. The other time window was 20-ms wide, ranging from 10 ms before the peak of the P3 component recorded from electrode FCz to 10 ms after the peak.

[2]http://www.fil.ion.ucl.ac.uk/spm/

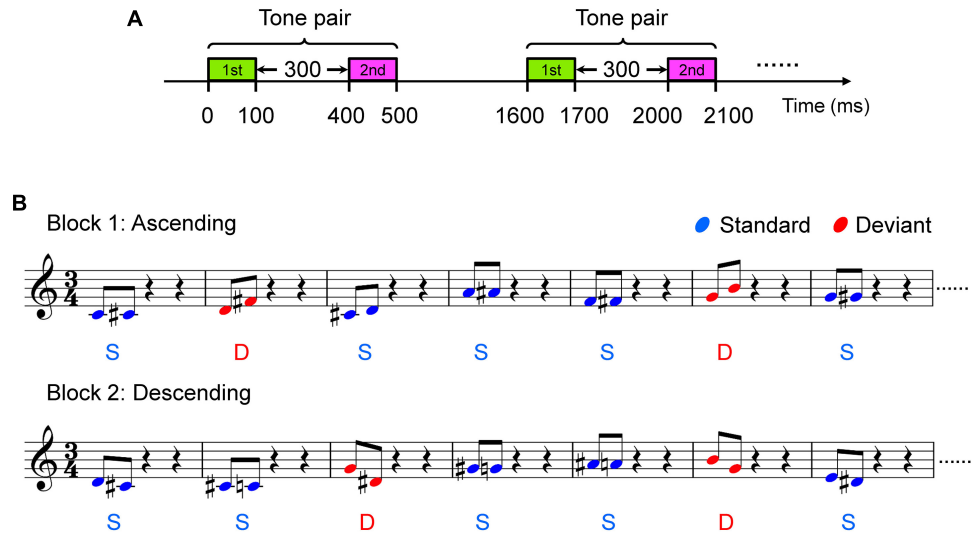

FIGURE 1 | Two blocks of oddball paradigm. **(A)** Illustration of tone pairs. **(B)** Illustration of block 1 and block 2. Sample stave for two blocks is shown. Tones in block 1 are clockwise, which means pitch of the second tone of each tone pair is always rising. Tone pairs in block 1 with shorter distance (pitch interval = one semitone, e.g. C4 – #C4) are standard stimuli and those with a larger distance (pitch interval = four semitones, e.g. C4 – E4) are the deviant stimuli. Tones in block 2 are counterclockwise, which means pitch of the following tone of each tone pair is always falling. Tone pairs in block 2 with shorter distance (pitch interval = one semitone, e.g. #C4 – C4) are standard stimuli and those with a larger distance (pitch interval = four semitones, e.g. E4 – C4) are deviant stimuli.

Behavioral Test

To assess the relationship between the observed brain activities and the behavioral abilities after the EEG collection was finished, we described the rules of the two types of blocks to each subject and then performed a behavioral test using E-Prime software. We instructed each subject to press the button "1" when they determined the pitch interval of the tone pairs to be short (i.e., the one-pitch interval), and press the button "2" when they perceived the pitch interval of the tone pairs to be large (i.e., the four-pitch interval).

Dipole Source Analysis

The localization of the dipoles generating the MMN and P3 activity was modeled using the BESA software package (Heuser-Link et al., 1992). The latency range where MMN responses were evident (510–560 ms) was selected for dipolar modeling. We first computed a 3D current source density (CSD) mapping with the grand average MMN. The CSD maps, expressed in $\mu V/cm^2$, were constructed by calculating the volume current flow out of the brain through the skull into the skin by means of the surface Laplacian operator (second spatial derivative of the voltage distribution in tissue). This method reduces the effects of volume conduction to the scalp potential and allows for better visualization of the approximate locations of intracranial generators that contribute to MMN. We modeled the MMN response by a bilateral dipolar source and then conducted a local autoregressive average (LAURA) distributed linear inverse solution at the peak of global field power (GFP) of MMN waveform using a lead field (solution space) with the value of regularization of 0.03%. LAURA depicts the degree of CSD brain activity within derived source regions, which allows us to show the source of MMN located in the left and right auditory cortex. When it comes to P3 source localization, we selected the time interval of 560–660 ms and performed principal components analysis (PCA) to determine three pairs of symmetrical regional sources were required to model the grand average P3 (criterion: explained variance >1%). Then, the model developed on the grand average of all subjects was applied to the individual data and we conducted LAURA distributed linear inverse solution at the peak of GFP of P3 waveform using a lead field with the value of regularization of 0.03%.

Dynamic Causal Modeling

Dynamic causal modeling (DCM) is an approach developed for connectivity analysis of functional magnetic resonance imaging (Friston et al., 2003). This method has been extended to magneto/encephalography (M/EEG) data (Garrido et al., 2007a; Boly et al., 2011). Most approaches to connectivity analysis of M/EEG data use functional connectivity measures, such as coherence, phase-synchronization or temporal correlations, which establish statistical dependencies between activities in two sources. Functional connectivity is useful, because it bases on the operational definition and is therefore independent of how the dependencies are caused (Garrido et al., 2007a). However, there are certain cases where causal interactions are the focus of interest. Here, DCM is particularly useful, because it uses the concept of effective connectivity. Effective connectivity refers explicitly to the influence one neuronal system exerts over another and can be estimated by perturbing the system and measuring the response by using Bayesian model inversion (Friston et al., 2003). In the context of EEG/MEG, DCM furnishes spatiotemporal, generative or forward models for evoked responses as measured with EEG/MEG (David et al., 2006;

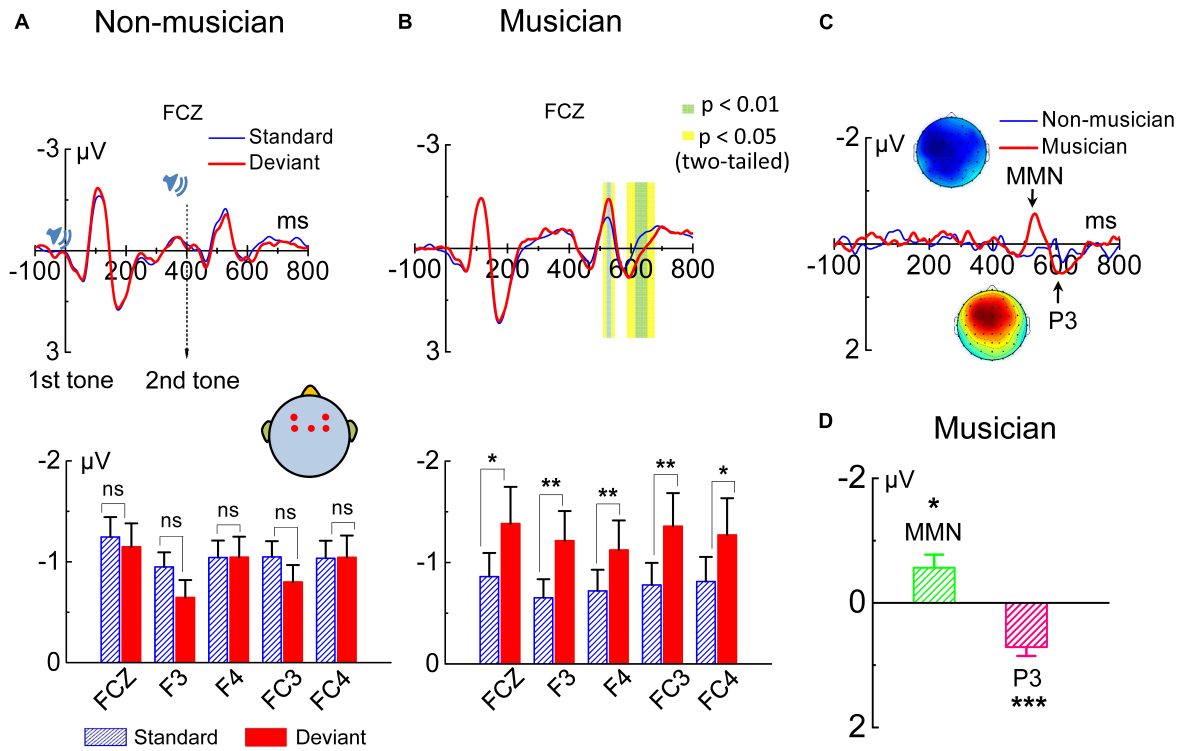

FIGURE 2 | Grand average event-related potential (ERP) at electrode FCz. ERPs evoked by standard and deviant tone pairs, as well as deviant-minus-standard difference waveforms are plotted for groups of non-musicians and amateur musicians. **(A)** No MMN evoked in the non-musician group ($p \geq 0.05$). **(B)** Amateur musician group extracted significant MMN amplitudes. Significant effects are marked with green and yellow areas in the waveforms. In the bar plots, significant effects are marked with asterisks (*$p \leq 0.05$, **$p \leq 0.01$). **(C)** Difference wave evoked in non-musicians (blue lines) and amateur musicians (red lines). ERP waveforms at the peak amplitude of MMN and P3 in the oddball paradigm of amateur musician group and the corresponding topographic map of MMN and P3 are shown. **(D)** Bar plots for MMN and P3 in the amateur musician group. Significant effects are marked with asterisks (*$p \leq 0.05$, ***$p \leq 0.001$).

Kiebel et al., 2006). DCMs for MEG/EEG use neural mass models (David and Friston, 2003) to explain source activity in terms of the ensemble dynamics of interacting inhibitory and excitatory subpopulations of neurons, based on the model of Jansen and Rit (Jansen and Rit, 1995). Briefly, DCM provides an account of the interactions among cortical regions and allows one to make inferences about system parameters and investigate how these parameters are influenced by experimental factors; furthermore, by taking the marginal likelihood over the conditional density of the model parameters, one can estimate the probability of the data, given a particular model (Garrido et al., 2007b). This is known as the marginal likelihood or evidence and can be used to compare different models. Early components of the ERP have been linked to exogenous bottom-up stimulus-bound effects, whereas late components have been related to endogenous dynamics involving top-down influences (Garrido et al., 2007b).

Source Reconstruction and Model Specification

We applied 3D source reconstruction analysis for choosing the prior source locations in the following DCM model specification. Normalization parameters were obtained using unified segmentation of the subjects' structural images (computerized tomography or T1 MRI) as implemented in the SPM software. Co-registration of electrode position and head model was performed for each subject prior to forward model computation. After the forward model was computed for each subject, the lead-field mapping of the cortical sources onto the measured signals was parameterized in terms of the location and orientation of each dipole source in the DCM (Garrido et al., 2008). **Supplementary Table 1** and **Supplementary Movie 1** show the coordinates for the locations of equivalent current dipoles (ECDs) in Montreal Neurology Institute (MNI) space (mm). The left and right primary auditory cortex (A1) were chosen as the cortical input stations for processing auditory information, both sides of temporal and frontal lobes were selected. By using these sources and prior knowledge about functional anatomy, we built a connectivity graph that featured an extrinsic input to the bilateral A1, which were connected to the corresponding ipsilateral temporal lobes, and both temporal lobes connected to the corresponding ipsilateral frontal lobes. Given this connectivity graph, specified in terms of its nodes and connections, we tested three models that differed in terms of the presence of reciprocal or recurrent connections: model F and model B had only forward and backward connections, respectively (**Supplementary Figures 1A, B**), while model FB had reciprocal connections, i.e., both forward and backward connections (**Supplementary Figure 1C**). In other words, model FB resembles recurrent dynamics or parallel bottom-up and

top-down processing, whereas model F and model B emulate a simple bottom-up and top-down mechanism, respectively.

We selected a time window of interest spanning 490–560 ms to perform identical analyses for each subject of amateur musicians in the preprocessing stage of DCM. We modeled each active source, namely, each node in the network, with a single ECD in a conventional electromagnetic forward model. This electromagnetic model employed boundary element head models (Fuchs et al., 2001), with homogeneous and isotropic conductivity as an approximation to the brain, cerebrospinal fluid, skull, and scalp surfaces. Subject-specific head models were obtained using the inverse spatial normalization of a canonical mesh for each subject. Then, we used a two-stage approach in statistical analyses in this research, firstly, Bayesian model selection (BMS) was used to optimize the network architecture underlying electrophysiological responses to auditory stimulation in amateur musicians. Secondly, quantitative connectivity analysis was performed, conditioned upon the best model selected in Bayesian model comparison, searching for effective connectivity of the amateur musicians respond to auditory stimulation (Boly et al., 2011).

Bayesian Model Selection

The Bayesian brain hypothesis uses Bayesian probability theory to formulate perception as a constructive process based on internal or generative models, a free-energy principle has been proposed recently that accounts for action, perception and learning, the brain is an inference machine that actively predicts and explains its sensations. This generative model is decomposed into a likelihood (the probability of sensory data, given their causes) and a prior (the *a priori* probability of those causes) (Friston, 2010).

Bayesian model selection is used to decide which model, amongst a set of competing models, best explains the data (Penny et al., 2004). Inversion of a specific DCM involves optimizing a model (m) which provides two important quantities: the free-energy bound on the model-evidence $p\left(y|m\right)$, used for model comparison, and the posterior or conditional density of the model parameters, $p\left(\theta|y,m\right)$. Specifically, DCM inversion corresponds to approximating the posterior probability of the parameters using variational Bayes (Friston et al., 2002). The aim is to minimize a free-energy bound on the log-evidence, with respect to a variational density, $q\left(\theta\right)$. When the free-energy is minimized; $q\left(\theta\right) = p\left(\theta|y,m\right)$ and the free-energy $F = -\ln p\left(y|m\right)$ approximates the negative marginal log-likelihood or negative log-evidence. After convergence, the variational density is used as an approximation to the desired conditional density and the log-evidence is used for model comparison. One often wants to compare different models and select the best before making statistical inferences on the basis of the conditional density. The best model, given the data, is the one with highest log-evidence $\ln p\left(y|m\right)$ (assuming a uniform prior over models). Given two models m1 and m2 one can compare them by computing their Bayes factor, i.e., the difference in their log-evidences $\ln p\left(y|m1\right) - \ln p\left(y|m2\right)$ (Garrido et al., 2007b).

In empirical or hierarchical Bayes models, the prior belief about the underlying causes of sensory input, $p\left(\theta\right)$, is optimized by higher hierarchical levels (i.e., higher-level brain areas) and provides top-down predictions on the most likely representations in lower levels. These "most likely" representations maximize the posterior belief or conditional density $p\left(\theta|y\right)$ of the causes of sensory data y. Bayes' rule defines the conditional density as $p\left(\theta|y\right) \propto p\left(\theta\right)p\left(y|\theta\right)$. This rule combines the top-down prior and a likelihood $p\left(y|\theta\right)$, which corresponds to the generative model used by the brain to predict its sensory input.

Quantitative Connectivity Analysis

We used the winning model (FB model, **Figure 5A**) from BMS above for final statistical analysis of the estimates of effective connectivity. In our DCMs, the effects of deviant stimuli (relative to standards) are modeled by scaling the effective connectivity in a trial-specific fashion. Although we tested for group differences in this (MMN-related) scaling, our primary interest was in differences in the underlying connection strengths mediated distributed responses to all stimuli. For analysis of quantitative connectivity, we compared the connectivity estimates (from the best model) by using paired sample *t*-tests, and then tested for differences in connection strength among the forward, backward, and lateral connections of the two hemispheres.

RESULTS

A Robust Mismatch Negativity Response Was Evoked in Amateur Musicians but Not in Non-musicians

Participants were allocated into an "amateur musician" group and a "non-musician" group according to whether they had obtained long-term musical learning, such as playing an instrument. The groups did not differ in age or the proportion of sexes. To investigate implicit memory, we used a classical auditory oddball paradigm in which implicit memory can be probed with the evoked MMN.

The grand-averaged ERPs in response to the auditory stimuli were calculated with recordings from the FCz electrode for each subject. Paired sample *t*-tests were performed at each sampling point throughout the whole epoch (−100 to 800 ms, one sampling point per 2 ms) for each subject in the amateur musician group and in the non-musician group. Results obtained from amateur musicians showed that the ERP responses to the standard and deviant stimuli differed significantly ($p < 0.05$, two-tailed) at two time windows: 518–546 ms (i.e., 118–146 ms after the onset of the second tone in the tone pair), and 586–666 ms (i.e., 186–266 ms after the onset of the second tone) but did not differ significantly outside these time windows (**Figure 2B**). However, the EEG data of the non-musicians showed no significant difference ($p > 0.05$, two-tailed) between the ERP responses elicited by standard and deviant stimuli (**Figure 2A**). We next used the time window selected from 515 to 545 ms post stimulus onset, i.e., 115–145 ms after the onset of the second tone in the tone pair for MMN analysis. The MMN amplitudes, calculated from the recordings from two pairs of electrodes on the left (F3 and FC3) and right (F4 and FC4) sides of the scalp, revealed that MMN could be evoked from the changes

in pitch interval in amateur musicians but not in non-musicians (**Figures 2A,B**). Different waves evoked by amateur musicians and non-musicians, as well as topographic map of MMN and P3 were shown in **Figure 2C**. Independent sample *t*-tests (two-tailed) showed that the amplitudes of the MMN component of the ERPs evoked by the amateur musicians were significant (**Figure 2D**). Given that this study was designed with a classically strict implicit memory retrieval paradigm, the MMN evoked by the changes in pitch interval evoked in the amateur musicians reflects their capacity for implicit memory retrieval.

To further investigate the MMN response, we analyzed the localization of the dipoles generating the MMN activity. The CSD mapping of MMN showed on the scalp surface a negative polarity over the frontocentral site and a positive polarity around the inferotemporal site (**Figure 4A**), indicating bilateral temporal generators accounting for MMN responses to tone pairs of four-pitch interval. Local autoregressive average (LAURA), a distributed source analysis, and dipole solution, a discrete source analysis, further confirmed that the generators of the MMN are located in the left and right temporal cortex in the musical group (**Figure 4A**). And the dipole strength of grand average MMN indicated a left hemisphere dominance of MMN in response to tone pairs of four-pitch interval, in line with the model in which left hemisphere being primary filling in the detailed pitch interval structure (Peretz, 1990; Warren, 2008).

A Robust P3 Response Was Evoked in Amateur Musicians

Surprisingly, the whole-head EEG recordings showed that the amateur musicians evoked a significant P3 component followed the MMN. Paired sample *t*-tests performed at each sampling point throughout the epoch (−100 to 800 ms, one sampling point per 2 ms) for subjects revealed that the ERPs of the amateur musicians evoked by the standard and deviant stimuli differed significantly at 586–666 ms (i.e., 186–266 ms after the onset of the second tone of the tone pair). The grand averages of the ERP waveforms in response to the standard and deviant stimuli over the 610–630 ms time window for electrode FCz across non-musicians and amateur musicians are shown in **Figures 2A,B**. Independent samples *t*-tests (two-tailed) were further performed for each of the two types of stimuli, and the results showed there was a significant difference between the ERP amplitudes evoked by the standard and deviant stimuli of the amateur musicians (**Figure 2B**); no significant difference was shown in non-musicians. Therefore, we concluded that significant P3 could be elicited in response to changes in the pitch interval under the classical oddball paradigm in amateur musicians but not in non-musicians (**Figures 2A,B**). Independent sample *t*-tests (two-tailed) revealed that the amplitudes of the P3 component of the ERPs evoked by the amateur musicians were statistically significant (**Figure 2D**).

Next, source localization of the dipoles generating the P3 activity was analyzed. The CSD mapping of P3 showed on the scalp surface a positive polarity over the frontocentral site and a negative polarity around the inferotemporal site (**Figure 4B**), indicating bilateral generators accounting for P3 responses to the tone pairs of four-pitch interval. LAURA showed that the generators of the P3 are located in the frontal lobe and insula (**Figure 4B**). Importantly, 3D source movie revealed the dipoles moved from the frontal lobe (**Figure 4B** and **Supplementary Movie 2**), which has been associated with unconscious attention (Stuss and Alexander, 2000; Polich, 2007; Axelrod et al., 2015) to the insula (**Figure 4B** and **Supplementary Movie 2**), which is known to be highly dependent on conscious attention to stimuli according to previous studies (Bekinschtein et al., 2009; Citherlet et al., 2019). Moreover, P3 has often been claimed to be a key signature of conscious perception (Babiloni et al., 2006; Del Cul et al., 2007; Dehaene and Changeux, 2011; Salti et al., 2012; Rutiku et al., 2015).

Amateur Musicians Showed Better Behavioral Performance Than Non-musicians

According to behavioral tests, the performance of amateur musicians was significantly better than that of non-musicians. Responses to the behavioral test by amateur musicians and non-musicians were recorded by E-Prime software. We tested the percentage of correct responses of subjects in two groups by using independent sample *t*-tests (two-tailed). The results showed that the performance of the two groups was significantly different, in which the percentage of correct responses by the amateur musicians was much higher than that by the non-musicians (**Figure 3A**). Moreover, Pearson's correlation analysis was performed to assess the correlation among the MMN, P3, and behavioral performance of the amateur musicians. The results showed that there was a significant correlation between ERP amplitudes and accuracy in the behavioral test (**Figure 3B**: $r = -0.506$, $p = 0.032$; **Figure 3B**: $r = 0.501$, $p = 0.034$).

Effective Connectivity From the Right Frontal Lobe to the Ipsilateral Temporal Lobe in Amateur Musicians

To reveal the detailed processing characteristics of implicit memory retrieval, we used DCM of the ERPs to quantify effective connectivity of the amateur musicians. Three models, differed in the areas and connections involved (**Figure 5**), were constructed and BMS was used to compare these three models. Fixed effects family level analysis showed that models including two frontal sources with both forward and backward connections could best explain the ERP responses in amateur musicians (**Figure 5A**). Then, we used the winning model, i.e., FB model, from BMS for the final statistical analysis of the calculation of effective connectivity. To further analyze the quantitative connectivity and compare the memory process between the two hemispheres, we analyzed the connectivity calculates from the best model using paired sample *t*-tests. The data indicated a significant difference on the backward connection from the frontal lobe to the temporal lobe of the right hemisphere compared to the backward connection from the frontal lobe to the temporal lobe of the left hemisphere (**Figures 5B,C**) and no difference between the two hemispheres on the forward or lateral connections (**Figures 5B,C**).

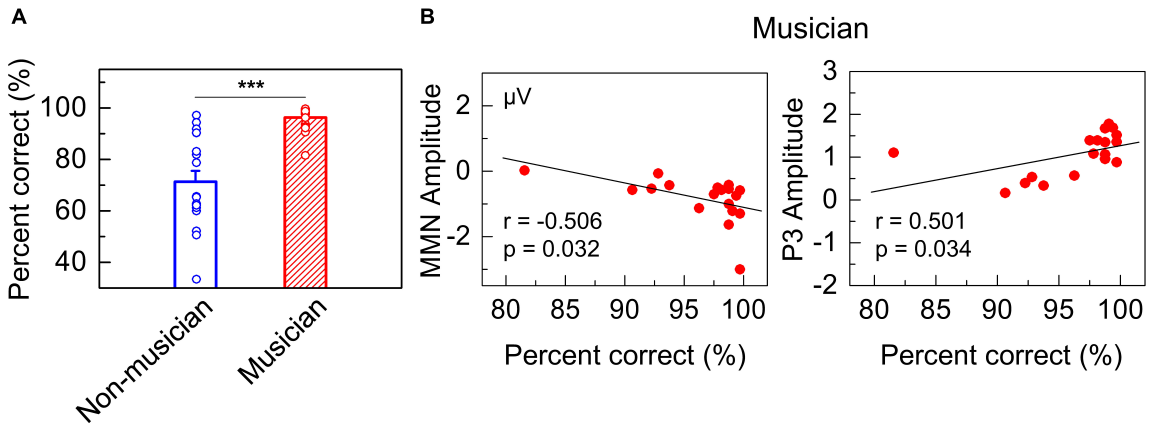

FIGURE 3 | Behavioral results. **(A)** Plots showing significant difference in percent of correct responses between groups of amateur musicians and non-musicians (***$p < 0.001$). **(B)** The left panel shows significant correlations between the percent of correct responses and MMN amplitude in amateur musicians. The right panel shows significant correlations between the percent of correct responses and P3 amplitude.

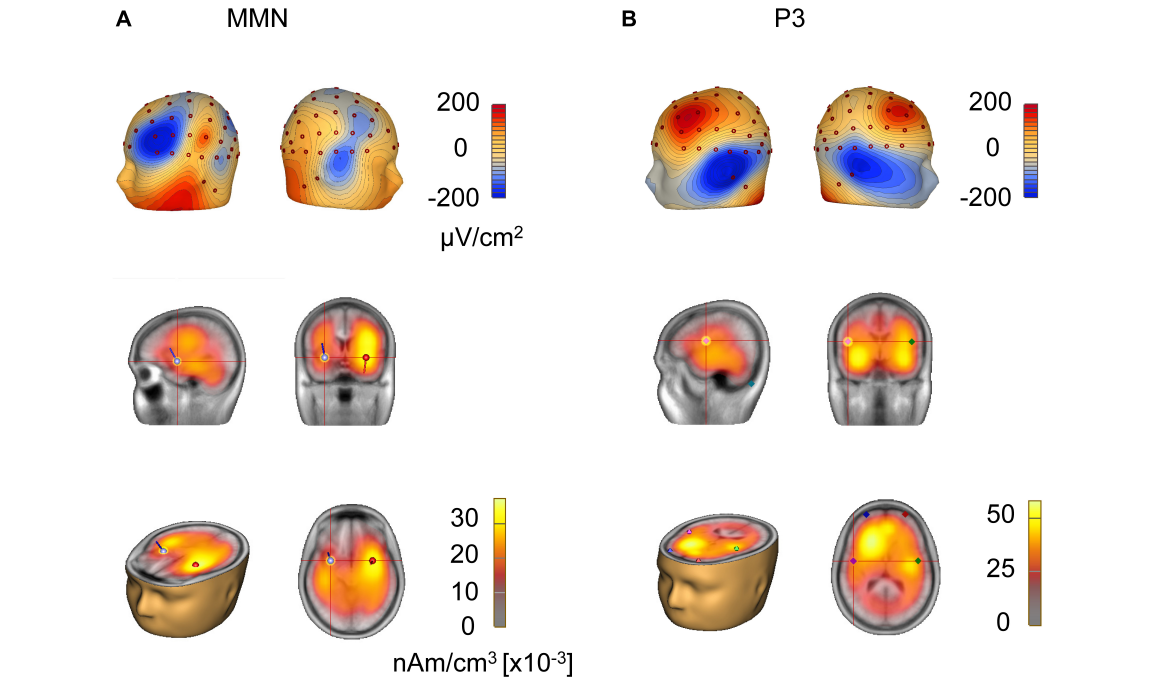

FIGURE 4 | Source analysis of MMN and P3. **(A)** Current source density topography at the peak of global field power of grand-averaged MMN in response to tone pairs of four-pitch interval. Source localization estimated by local autoregressive average and dipole solution of MMN in response to tone pairs of four-pitch interval. **(B)** Current source density topography at the peak of global field power of grand-averaged P3 in response to tone pairs of four-pitch interval. Source localization estimated by local autoregressive average and dipole solution of P3 in response to tone pairs of four-pitch interval.

DISCUSSION

The present study was carried out in healthy subjects at the temporal dimension during implicit memory retrieval. Our results demonstrate a robust P3 component during implicit memory retrieval of musical pitch interval in musicians, which is believed by many to be an indicator of involuntary consciousness accompanying the implicit memory retrieval. Specifically, our results suggest that implicit and explicit memories may not necessarily have to be clearly differentiated by whether consciousness is involved and that aspect of memory processing, such as top-down process, might be considered as an effective factor in defining types of memory. Our study raises the possibility that consciousness, to some extent, may not be associated with the definition of memory. In our study, EEG was recorded under a traditional oddball paradigm to

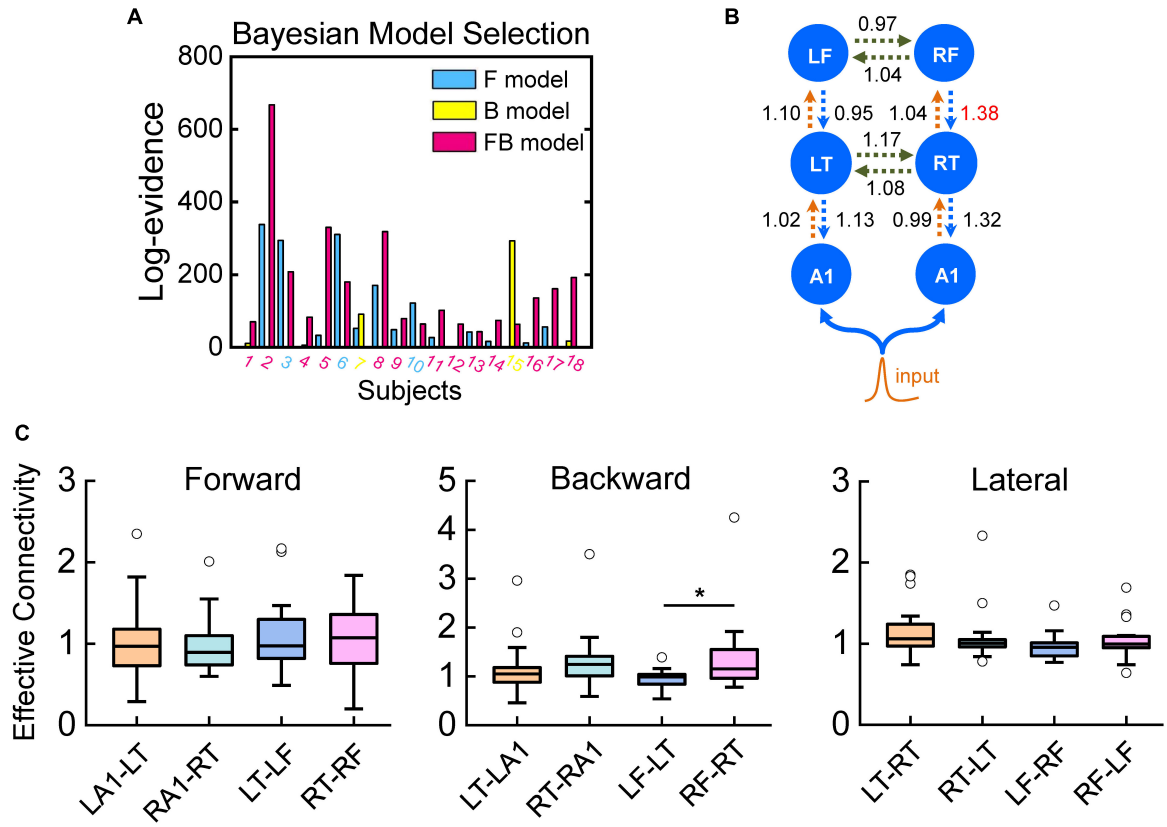

FIGURE 5 | Quantitative effective connectivity. Bayesian model selection among DCMs for the three models, F, B, and FB, expressed relative to a DCM in which no connections were allowed to change (null model). The graphs show the free energy approximation to the log-evidence. **(A)** Log-evidence for models F, B, and FB for each amateur musician (relative to the null model). The diamond attributed to each subject identifies the best model on the basis of the subject's highest log-evidence. Log-evidence at the group level, i.e., pooled over subjects, for the three models. **(B)** Effective connectivity of the FB model. **(C)** In the bar plots, significant difference on the backward connection from the frontal lobe to the temporal lobe of the right hemisphere compared to the backward connection from frontal lobe to temporal lobe of the left hemisphere and no difference between the two hemispheres on the forward and lateral connections.

directly compare the auditory processing of different musical pitch intervals in amateur musicians and non-musicians. We provided a behavioral test to the two groups of participants and assessed their performance in terms of the percentage correct responses. The electrophysiological results showed that large MMN and P3 amplitudes could be elicited in subjects with long-term musical learning but not in participants without musical training for either stimulus block (**Figures 2A,B**). Furthermore, the amateur musicians behaviorally outperformed non-musicians (**Figure 3A**), which is associated with the MMN component evoked by the oddball paradigm among the amateur musicians. The behavioral findings demonstrate a significant correlation with EEG results, i.e., larger amplitudes were correlated with higher accuracy in the behavioral test (**Figure 3B**). These results are consistent with previous studies in which amateur musicians performed better than non-musicians when detecting speech in noise and demonstrate enhanced subcortical auditory and audiovisual encoding of speech and music sounds (Musacchia et al., 2007; Song et al., 2012). Additionally, brain electrical source analysis of the P3 component evoked by amateur musicians revealed that the dipoles moved from the frontal lobe to the insula (**Figure 4B** and **Supplementary Movie 2**), which is known to be highly dependent on conscious attention to stimuli according to previous studies (Bekinschtein et al., 2009; Citherlet et al., 2019). Notably, analysis of the P3 component suggests that the implicit memory retrieval of musical pitch intervals in this study may involve unconscious access. The effective connectivity obtained by DCM analysis also reveals a significant increase in backward connectivity, namely, top-down processing from the right frontal lobe to the ipsilateral temporal lobe, in amateur musicians (**Figures 5B,C**). This is in line with some evidence from auditory and visual studies of humans and animals supporting that explicit memory retrieval is under the active executive control of top-down processes from the prefrontal cortex (Hasegawa et al., 1998; Tomita et al., 1999; Miyashita, 2004; Kostopoulos and Petrides, 2016; Risius et al., 2019). This suggests that implicit and explicit memories may share a similar underlying neurocognitive mechanism. Altogether, our study presents a case of involvement of involuntary consciousness during implicit memory retrieval and suggests a potential challenge to the classical definition of implicit memory.

Implicit Memory Retrieval of Musical Pitch Interval May Involve the Brain Circuit Associated With Involuntary Consciousness

Traditionally, when we refer to implicit memory, we mean memory defined as obtained knowledge that is not available to conscious access (Schacter and Graf, 1986). For instance, learning to ride a bicycle initially involves conscious attention to one's body and the bicycle. Later, riding eventually becomes an automatic activity, which can be regarded as implicit memory shaped through learning and does not necessarily involve awareness of the memory (Kandel, 2006). Analogous to the implicit memory of riding a bicycle, amateur musicians who undergo long-term musical learning is a good model for studying implicit memory. In our study, significant MMN responses can be elicited in amateur musicians (**Figure 2B**), which has been widely used as an effective electrophysiological signature for studies of early auditory processing (Luo et al., 2006; Gu et al., 2012; Wang et al., 2013; Guo et al., 2018). It is also regarded by many as a probe of implicit memory. In our experiment, the evocation of MMN by amateur musicians in the preattentive stage suggests that long-term musical learning promotes the generation of implicit musical memory in their memory storage systems. Surprisingly, our neurophysiological measures reveal a significant P3 amplitude following the MMN in amateur musicians (**Figure 2B**). P3 is a positive-going component of evoked-potential waveforms that has been associated with the processing of unexpected events and was first reported in 1965 (Sutton et al., 1965). P3 is elicited most commonly in the context of the auditory oddball paradigm, where it can be used as an index of the involuntary shift of attentional resources toward novel stimuli and can be evoked under attending or ignoring situations (Ritter et al., 1968; Kok, 2001; Horváth et al., 2008). Some studies have proposed that MMN, especially frontal MMN, is associated with P3, indicating involuntary attention switching (Schröger, 1996; Rinne et al., 2000). Because our results show a salient P3 component evoked among the amateur musicians under the oddball paradigm for the retrieval of implicit memory, we propose that the attention of amateur musicians was drawn involuntarily by the deviant stimuli. Non-musicians apparently could not distinguish the rare stimuli, as suggested by the absence of P3 component, which depends on the ability to process task-relevant stimuli and reflects event classification *via* the correlation of attention and the working memory network (Kok, 2001). In addition, source localization of the dipoles generating the activity of the P3 component modeled by the BESA software package reveals the dipoles moving from the frontal lobe (**Figure 4B** and **Supplementary Movie 2**), suggesting an unconscious attention (Stuss and Alexander, 2000; Polich, 2007; Axelrod et al., 2015) is associated with the insula (**Figure 4B** and **Supplementary Video 1**), which is known to be highly dependent on conscious attention to stimuli (Bekinschtein et al., 2009; Citherlet et al., 2019). Thus, we speculate that there is a transition from unconsciousness to consciousness during the P3 component of implicit memory and that the implicit memory retrieval of musical pitch interval may involve the consciousness. Accumulating evidence from studies on explicit memory indicates that an intact hippocampus is necessary for rapid associative learning with and without consciousness (Henke, 2010). In this vein, our study on implicit memory retrieval provides ERP evidence supporting the view that consciousness may be an inadequate criterion for differentiating types of memory.

Implicit Memory and Explicit Memory May Share an Analogous Underlying Neurocognitive Characteristic: Top-Down Processing

Top-down regulation is an experience-dependent process that originates from the prefrontal cortex, carrying an abundant amount of prior knowledge and transmitting information synthesized from experience that facilitates an individual's interpretation of input information (Tomita et al., 1999; Lee and D'Esposito, 2012). Moreover, such stored information provides context and meaning to sensory inputs, which is central to high-level cognition of basic auditory processing and visual recognition (Sohoglu et al., 2012; Gilbert and Li, 2013). A previous study in speech perception suggests that a top-down mechanism would be reflected with abstract computations in the inferior frontal gyrus (IFG) being modulated before sensory-related processing in the superior temporal gyrus (STG) (Sohoglu et al., 2012). Behavioral evidence from recent studies have shown that musical learning also has a close relationship with top-down pathway and suggest that top-down regulation is involved in the formation of music-related memory in the auditory processing (Kraus and Chandrasekaran, 2010; Strait et al., 2010). Since explicit memory is characterized by knowledge that involves conscious recollection, recall, or recognition (Ettlinger et al., 2011), evidence from the auditory and visual studies of humans and animals supports the notion that explicit memory retrieval is under the executive active control of top-down processes. Many studies on memory research have reported that episodic memory, which is classified as explicit memory, is associated with conscious encoding (Rombouts et al., 1997; Henke et al., 1999; Staresina and Davachi, 2009) but may not involve consciousness (Henke et al., 2003a; Degonda et al., 2005). Therefore, one model proposes that different types of memory are distinguished according to the processing operations involved rather than by consciousness (Henke, 2010). However, to the best of our knowledge, the majority of previous studies on memory processing are based on explicit memory research (Tomita et al., 1999; Boly et al., 2011), little work has been done from the perspective of implicit memory retrieval to examine whether implicit memory and explicit memory can be distinguished based on consciousness. In the present study, measures of effective connectivity show distinguishable backward connectivity from the right frontal lobe to the ipsilateral temporal lobe in amateur musicians (**Figure 5**), which demonstrates that top-down processing is involved in the implicit memory retrieval of musical pitch intervals. These findings are consistent with those indicating the involvement of top-down processing in the retrieval of

explicit memory (Hasegawa et al., 1998; Tomita et al., 1999; Miyashita, 2004). Combining the above points, we further suggest that, to some extent, implicit and explicit memory may share an analogous underlying top-down neurocognitive mechanism. Indeed, all of these results clearly demonstrate that long-term musical learning induces brain plasticity, which accounts for the activation of top-down processing (i.e., backward frontotemporal connectivity). Additionally, in the present study, the better behavioral performance (**Figure 3A**) in the selection of pitch interval, as well as the more robust MMN response elicited in amateur musicians than in non-musicians, suggests that amateur musicians have prior knowledge of the pitch interval and that musical training can induce their abilities to detect the pitch interval of a tone pair *via* top-down processing. Therefore, we suggest that top-down signals and prior knowledge-related and higher-order recognition processing participate in the retrieval of the implicit memory of pitch interval.

Bayesian Inference and Predictive Coding in the Brain: The Mechanism Underlying the Automatic Detection of Changes

Bayesian inference has been proposed as a basic principle for brain function, which is based on an internal generative model used by the brain to predict sensory input, that comprises a distribution over sensory data given an external cause (the sensory data likelihood) and a prior distribution over different causes (Summerfield et al., 2006; Joos et al., 2014). The predictive coding framework is a well-known hypothesis of the mechanism of human sensory perception. The central assumption in predictive coding theory is that the activity in the nervous system reflects a process of matching internally generated predictions, which anticipates the forthcoming sensory environment, to external stimulation (Rao and Ballard, 1999; Heekeren et al., 2008; Rauss and Pourtois, 2013). Predictive coding, under which the brain is regarded as a hierarchically organized cortical system, is a general theory of perceptual inference, and recently, it has been proposed as the mechanism underlying the generation of the MMN component and has been formulated in terms of empirical Bayesian models of perceptual learning and inference (Näätänen et al., 2007; Garrido et al., 2009). As we mentioned above, the MMN component has been identified as a typical neurobiological marker for error (uncertainty or unexpectedness) detection caused by deviant inputs. Previous studies have also shown that recurrent dynamics generate evoked brain responses in cortical networks, and feed-forward connectivity is sufficient to generate early ERP components; conversely, late components are mediated by backward connections (Garrido et al., 2007a; Boly et al., 2011). *Via* the results obtained from BMS, we found that the best model includes modulations of both forward and backward connections (FB model, **Figure 5A**). Our results support and extend findings showing that a frontotemporal network is involved in generating mismatch responses and that this generation entails an interaction between top-down and bottom-up exchanges between cortical sources, in line with the results from other studies (Kiebel et al., 2009).

Next, the Bayesian brain model proposes that our brain works in a Bayesian way under the free-energy principle, which asserts that any adaptive change made by a biological system or organism must minimize its free energy (i.e., reduce environmental uncertainty, unexpected or unpredicted sensations) (Edwards et al., 2012). In this model, the Bayesian brain can be conceptualized as a probability machine that always makes predictions about the world and updates them based on what it senses (Friston, 2010). Thus, we suggest that the brains of amateur musicians can use prior knowledge (implicit memory of musical pitch interval) to predict the incoming sensory inputs to reduce the uncertainty of the environment (i.e., prediction error). Neuronal activity reflects attempts to minimize or reduce prediction error (i.e., uncertainty) to estimate the most likely cause of the input and represent the states of the world according to the free-energy principle. Repeated stimuli reduce the prediction error from bottom-up regulation, while the detection of deviant stimuli may lead to the increase of the prediction error. In the current study, we propose that due to long-term musical learning, amateur musicians possess the implicit memory related to musical elements. Such participants can not only determine that the stimuli are being presented in pairs but also recognize the inner rule of the stimuli, i.e., pitch interval. In this circumstance, the predictive top-down signals from the frontal lobe to the temporal lobe associated with two characteristics of the stimuli (i.e., tone pairing and pitch interval) were expected by the prediction units of the amateur musicians according to information about the stimuli they had previously acquired. If the predicted stimulus is consistent with the incoming stimulus, the prediction error will gradually be minimized based on the free-energy principle. Otherwise, when a deviant stimulus with a pitch interval different from that of the predicted stimulus is presented, after the real stimuli heard from the headphone are compared to the sounds predicted by the amateur musician's prediction error units, the prediction error will increase, which will result in changes in the amplitude, direction and position of the dipole in the musician's brain. Then, the appearance of the MMN component of the ERP, a marker of automatic error detection, will be elicited because of the failure to minimize surprise, which leads to an increase in entropy in the brain system; and the P3 component of the ERP, an index of involuntary attention switch, is elicited. While non-musicians can only perceive the paired tone rule, they are unable to distinguish the difference in the pitch interval between the standard and incoming deviant stimuli, and their prediction error will be unchanged. Thus, an MMN cannot be elicited by the deviant stimuli in non-musicians in the present paradigm.

Amateur Musician Is a Good Model for Studying Implicit Memory

As we all know, music can move us, and musical learning plays a significant role in various respects of human hearing skills as well as different ages. For instance, in terms of infancy, active music classes in infancy enhance musical,

communicative, and social development (Gerry et al., 2012). Studies of children showed that musical learning during early childhood improves the neural encoding of speech in noise; besides, speech segmentation, pre-attentive processing of syllabic duration are directly facilitated by musical training (Chobert et al., 2012; Strait et al., 2012; Virtala et al., 2012; Francois et al., 2013). Moreover, adult research has examined brainstem encoding of linguistic pitch and that musicians show more robust and faithful encoding compared with non-musicians (Wong et al., 2007). Not only functional advantages but also structural changes have occurred in the brain of musicians, such as enlarged gray and white matter, and better developed cognitive function of left temporal correlated with verbal memory in musicians (Chan et al., 1998; Gaser and Schlaug, 2003; Chobert et al., 2011; Zatorre et al., 2012). Interestingly, we can hardly be surprised, meanwhile, that music lessons improve children's IQ (Schellenberg and Hallam, 2005), given that they will nourish general faculties such as memory, coordination, and attentiveness that they will nourish general faculties such as memory, coordination as well as attentiveness. Additionally, music skills have also been found to correlate significantly with both phonological awareness and reading development (Anvari et al., 2002).

Shepard tone used in the current research was generated by Shepard in 1964; each tone consisted of many sinusoidal components locked at successive intervals of an octave and sounded simultaneous. We used this type of stimulus to enhance the challenge for participants in the processing of different pitch intervals. In our study, EEG results showed that the larger pitch intervals, namely, deviant stimuli, can elicit significant MMN responses in amateur musicians, which is a component of ERP indicating automatic change detection (Näätänen et al., 2007). However, no MMN responses could be evoked by participants without long-term musical learning. We can therefore propose that long-term musical learning induces adult's automatic ability of processing pitch interval, and such capacity can be integrated into the existent automatic abilities. Consistent with previous studies, amateur musicians perform better than non-musicians when detecting speech in noise environment, as well as enhanced subcortical auditory and audiovisual encoding of speech and music sounds (Musacchia et al., 2007; Song et al., 2012). Additionally, previous research, for instance, the auditory brainstem responses when listening to musical intervals, has demonstrated results consistent with our study (Lee et al., 2009). Apart from this, the pianists also show increased neural activity (measured by magnetic source imaging) in the auditory cortex in response to hearing piano notes (Pantev et al., 1998; Brunelliere et al., 2009). Thus, we propose that long-term musical learning induces an adult's ability of automatic pitch interval processing, and such capacity can be integrated into the automatic detection of implicit memory.

Neural Correlate of Consciousness

Implicit memory retrieval of musical pitch interval in the current study seems to be highly related to the access neural correlate of consciousness. Ned Block describes how access neural correlate of consciousness (NCC) are distinct from phenomenal NCC: access conscious content is information about which is "broadcast" in the global workspace, while phenomenally conscious content is as different experience of red and green (Block, 2005). In other words, access conscious contents information about which is made available to the "consumer" systems of the brain: such as memory system, voluntary direction of attention system, perceptual categorization system, and more generally, system of rational control of action (Block, 2005). Two theories about neural basis of consciousness are put forward. One theory is Recurrent Processing Theory (RPT) (Lamme and Roelfsema, 2000; Lamme, 2006, 2010) and the other one is Global Neuronal Workspace Theory (GNWT) (Dehaene et al., 2006; Dehaene and Changeux, 2011). According to RPT, all perceptual organization required for vision of consciousness is achieved by the visual cortex and the frontal cortex has only modulatory influence to some extent. According to the GNWT, however, the dorsolateral prefrontal cortex (DLPFC) plays a critical role in mediating the conscious contents, at least in conscious "access" to the content information (Northoff and Lamme, 2020). In addition, GNWT with its focus on access rather than phenomenal consciousness points at the later brain activity (P300, more specifically P3b as observed in the present study) is regarded as the key signature of "global ignition," which becomes available of sensory information for other brain areas, and access to consciousness (Sergent et al., 2005; Dehaene and Changeux, 2011). There is no unchallenged best hypothesis on P3b (Verleger, 2020) and more studies on different hypotheses needs to be tested against each other. The relationship between P3 and consciousness, as the case in our present study, requires more investigation in future study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Biomedical Research Ethics Committee of the University of Science and Technology of China. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LC and X-YL designed the research. X-YL and X-TG performed the research. X-YL, Z-HG, and X-DW analyzed the data. LC, X-YL, H-WL, J-WS, and MW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.902175/full#supplementary-material

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Environmental Enrichment Reverses Maternal Sleep Deprivation-Induced Anxiety-Like Behavior and Cognitive Impairment in CD-1 Mice

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Preclinical studies have clearly indicated that offspring of mothers who suffered sleep deprivation during pregnancy exhibit anxiety, depression-like behaviors, and cognitive deficits. The cognitive impairment induced by maternal sleep deprivation (MSD) is currently poorly treated. Growing evidence indicates that an enriched environment (EE) improves cognition function in models of Alzheimer's disease, schizophrenia, and lipopolysaccharide. However, the effects of EE on hippocampal-dependent learning and memory, as well as synaptic plasticity markers changes induced by MSD, are unclear. In the present study, pregnant CD-1 mice were randomly divided into a control group, MSD group, and MSD+EE group. Two different living environments, including standard environment and EE, were prepared. When male and female offspring were 2 months, the open field test and elevated plus maze were used to assess anxiety-like behavior, and the Morris water maze was used to evaluate hippocampal learning and memory. Western blotting and real-time fluorescence quantitative polymerase chain reaction were used to detect the expression of brain-derived neurotrophic factor and Synaptotagmin-1 in the hippocampus of offspring. The results revealed that MSD-induced offspring showed anxiety-like behaviors and cognitive impairment, while EE alleviated anxiety-like behavior and cognitive impairment in offspring of the MSD+EE group. The cognitive impairment induced by MSD was associated with a decreased brain-derived neurotrophic factor and an increased Synaptotagmin-1, while EE increased and decreased brain-derived neurotrophic factor and Synaptotagmin-1 in the hippocampus of mice from the MSD+EE group, respectively. Taken together, we can conclude that EE has beneficial effects on MSD-induced synaptic plasticity markers changes and can alleviate anxiety-like behaviors and cognitive impairment.

Keywords: maternal sleep deprivation, learning and memory, BDNF, synaptotagmin-1, enriched environment

INTRODUCTION

Sleep is a conservative behavior of mammals. Adequate sleep is conducive to energy recovery, removal of toxic substances, and consolidation of learning and memory (Xie et al., 2013). However, in modern society, lack of sleep has become a prevalent phenomenon in many populations, especially for women during pregnancy (Sedov et al., 2018). Epidemiological studies have shown that about half of pregnant women complained about their sleep, which was characterized by decreased total sleep time, sleep efficiency, rapid eye movement sleep, and slow-wave sleep (Wilson et al., 2011). Notably, the abnormal sleep pattern in the third trimester of pregnancy has been ascribed to conditions such as nocturia, nausea, discomfort from fetal movements, difficulty in assuming usual sleep positions, back pain, and hormonal oscillations, and can lead to chronic sleep deprivation (Izci-Balserak et al., 2018). Concurrently, the third trimester of pregnancy is also a critical time for fetal brain and center nervous system development (Micheli et al., 2011). Clinical studies have found that chronic sleep deprivation not only increases the risk of psychiatric disorders in mothers but also has a series of adverse effects on the offspring (Chang et al., 2010). Due to ethical limitations, the underlying mechanisms can be explored through animal models of sleep restriction during pregnancy.

Similarly to many animal models of early life stress, the maternal sleep deprivation (MSD) model affects fetal hippocampal synapse development by disrupting the intrauterine environment (Entringer et al., 2012). Previous studies have found that MSD-induced rats showed hippocampal-dependent memory impairment, which was associated with decreased hippocampal neurogenesis and increased levels of pro-inflammatory markers due to microglial activation (Zhao et al., 2014). It is known that hippocampal synaptic plasticity indicated with long-term potentiation (LTP) is a cellular mechanism underlying information processing and memory formation (Yang et al., 2021). The offspring of mothers subjected to sleep deprivation at different stages of pregnancy have been found to exhibit anxiety, depressive-like behaviors, and cognitive deficits, accompanied by impaired LTP and basal vesicle transmission in the CA1 region of the hippocampus (Peng et al., 2016). Brain-derived neurotrophic factor (BDNF) and Synaptotagmin-1 (Syt-1) are two important synaptic plasticity markers, which have been experimentally confirmed to be associated with cognitive impairment induced by early life stress (Thome et al., 2001; Leal et al., 2015). BDNF, a main neurotrophin in mammals' hippocampus, is essential for synaptic transmission and regulates dendritic arborization and LTP, while Syt-1, a Ca^{2+}-dependent synaptic protein, can bind with SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex and directly interacts with SNAP-25 (synaptosomal-associated protein of 25 kDa) on a presynaptic membrane to facilitate neurotransmitter release (Spriggs et al., 2019; Chanaday et al., 2021). Collectively, gestational sleep deprivation causes emotional and cognitive dysfunction in offspring, but whether the mechanisms of cognitive dysfunction involve changes in synaptic plasticity markers is unclear.

The concept of an enriched environment (EE) was proposed by Hebb in 1947 and is a simple and effective method to improve cognitive deficits (Alwis and Rajan, 2014). EE is an experimental paradigm that allows mice to receive sensory, motor, and social stimulation by placing them in larger devices equipped with a variety of toys and running wheels (Yu et al., 2020; Zhang et al., 2021). EE has been found to affect cell survival, neurogenesis, synaptogenesis, and dendritic morphology in the hippocampus (Wang et al., 2020). Several findings have indicated that EE improves activity-dependent synaptic plasticity, LTP, social interaction, and spatial learning and memory in models of Alzheimer's disease, brain injury, Parkinson's disease, and schizophrenia (Murueta-Goyena et al., 2019). Our lab has also shown that long-term EE attenuates the exacerbated age-related cognitive impairment induced by lipopolysaccharide exposure during pregnancy (Zhuang et al., 2021).

In this study, we investigated the effects of EE on anxiety and spatial learning and memory in MSD-induced male and female offspring *via* increased dwelling space containing novel toys at the end of lactation. This study provides evidence for the establishment of EE as an additional therapy option for improving brain functioning in offspring who have suffered from MSD.

METHODS

Animals

Both male and female CD-1 mice (8 weeks) were purchased from Beijing Vital River Laboratory Animal Device Co., Ltd. (SPF grade). The animals were housed in individual cages maintained at a temperature of 22–25°C, a 12 h dark-light photoperiod, and 60%–70% relative humidity. Food and water were available ad libitum. All procedures were carried out in compliance with the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Experimental Protocols

The male and female CD-1 mice were acclimated to the new environment for 2 weeks, and were then paired at a ratio of 1:2. The 30 female mice with vaginal smear were selected and randomly assigned to one of the three following groups: (1) control group (n = 10), (2) MSD group (n = 10), and (3) MSD+EE group (n = 10). Pregnant mice of the MSD and MSD+EE groups were put into the BW-NSD404 sleep deprivation machine (Shanghai Bio-will Co., Ltd.) during the third period (GD15-GD21) of pregnancy from 12:00 to 18:00. The sleep deprivation machine ensured that mice remained awake during deprivation by the continuous work of the running belt and the speed of the running belt was set to 0.5 m/min. Concurrently, the pregnant mice of the Control group were put into the other BW-NSD404 sleep deprivation machine at a speed of 0 m/min. The mice from the three groups lived in the same environment during and after sleep deprivation. Mice had free access to food and water throughout the sleep deprivation period. After weaning, the offspring from the MSD+EE group (n = 15) were raised in larger cages (52 × 40 × 20 cm) with 7–8 mice/cage

containing various colorful toys, including platforms, a wood shelter, running wheels, ladders, and plastic tunnels. Objects were changed twice a week. The offspring from the MSD group (n = 15) and the Control group (n = 15) were raised in standard cages (36 × 18 × 14 cm) with three mice/cage without objects. At 2 months of age, all offspring were examined by behavioral and molecular experiments (**Figure 1**).

Open Field Test

In the open field test (OFT), mice were gently placed in the center of a black wooden box (50 cm × 50 cm × 25 cm) for 5 min, and exploratory behavior was automatically recorded by the ANY-maze video tracking system (Stoeling, USA). Time spent in and the number of entries into the central area and total distance were recorded by ANY-maze software. After each test, the arena was cleaned with 75% alcohol to avoid the interference of odor.

Elevated Plus Maze

The elevated plus maze (EPM) consisted of two open arms and two closed arms arranged at right angles. The height of the maze was about 50 cm above the ground. At the beginning of the experiment, mice were put into the central area of the maze with their head facing the open arms. The number of entries and the time spent in each arm were recorded for 5 min by the ANY-maze video tracking system. After the recording, the maze was wiped with 75% alcohol to eliminate the odor of the mouse.

Morris Water Maze

The Morris water maze (MWM) was used to assess the spatial learning and memory abilities of mice. The protocol used in this study was similar to those described previously (Wu et al., 2020). The test was divided into two parts: a learning phase and a memory phase. During the learning phase, mice were trained in a circular black pool (diameter 120 cm, height 30 cm) over four trials per day for seven consecutive days to find the hidden platform. Mice were allowed to rest on the platform for 30 s if they failed to find the hidden platform within 60 s. During the memory phase, a probe trial task was performed after the hidden platform had been removed. All trials were recorded and analyzed using the ANY-maze tracking system.

Western Blotting

The Western blotting procedure was performed as previously described (Zhuang et al., 2021). Hippocampal tissue was lysed in RIRA lysis buffer, and protein concentrations were determined using a BCA Protein Assay Kit. The protein samples were electrophoretically separated and then blotted onto nitrocellulose membranes. Protein levels were determined *via* incubation against antibodies of BDNF (1:1,000; Abcam, Cambridge, UK) and Syt-1 (1:1,000; Bioss, Beijing, China). Bands were visualized by enhanced chemiluminescence and quantified using ImageJ software.

Real-Time Fluorescence Quantitative Polymerase Chain Reaction

Total RNA was extracted from the hippocampal tissue by adding Trizol lysate. The purity of the extracted RNA was assessed using a spectrophotometer. RNA was reverse-transcribed to cDNA using the ReverAidTM First-Strand cDNA Synthesis Kit. The transcripts were amplified by quantitative real-time polymerase chain reaction. The reaction system consisted of 5 μl of 2× SYBR Green Mixture, 1 μl of upstream primer, 1 μl of downstream primer, 1 μl of cDNA, and 2 μl of RNase Free water. The reaction conditions were as follows: a single cycle of pre-denaturation at 95°C for 1 min, and a total of 40 cycles at 95°C for 20 s and 60°C for 1 min. The primer sequence is shown in **Table 1**.

TABLE 1 | Primer sequences.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
β-actin	AGTGTGACGTTGACATCCGT	TGCTAGGAGCCAGAGCAGTA
BDNF	TTACTCTCCTGGGTTCCTGA	ACGTCCACTTCTGTTTCCTT
Syt-1	GTCCTTCTAGTCGTGACCTG	GCCTGATCCTTCATGGTCTT

Data Analysis

All values are expressed as the mean ± standard error of the mean. Repeated-measure analysis of variance (ANOVA) was used to analyze data from the MWM test. Data of anxiety-like behaviors, cognitive cognition, and synaptic plasticity markers were analyzed using a two-way analysis of variance with Tukey's least-significant difference *post-hoc* test to compare differences between the three groups. Differences were considered significant at $P < 0.05$. All data analyses were performed using GraphPad 8.0.

RESULTS

Environmental Enrichment Reverses Anxiety-Like Behavior Induced by Maternal Sleep Deprivation

First, we evaluated the effect of MSD on anxiety-like behaviors using the OFT and EPM, and assess the potential therapeutic effects of EE. Two-way ANOVA revealed a significant treatment effect for center time ($F_{(2,84)}$ = 16.97, $P < 0.01$; **Figure 2A**) and number of the center entries ($F_{(2,84)}$ = 40.42, $P < 0.01$; **Figure 2C**) among the three groups during the OFT. The *post hoc* analysis confirmed that the mice from the MSD group exhibited more anxiety-like behaviors than the control group (Ps < 0.05), and EE reversed this abnormal mental state (Ps < 0.05). Similarly, a two-way ANOVA revealed significant between-group differences in open arms time ($F_{(2,84)}$ = 14.69, $P < 0.01$; **Figure 2B**) and number of the open arms entries ($F_{(2,84)}$ = 41.23, $P < 0.01$; **Figure 2D**) during the EPM. *Post hoc* analysis revealed that EE normalized the anxiety-like behavior associated with MSD (Ps < 0.05). There were no differences in the total distance among the three groups during the OFT and EPM, which indicated that MSD did not impair the motor ability of offspring (**Figures 2E,F**). Collectively, the OFT and EPM results indicated that EE had a healing effect on anxiety-like behaviors caused by MSD.

FIGURE 1 | Timeline of the experiment (see "Experimental Protocols" Section for details).

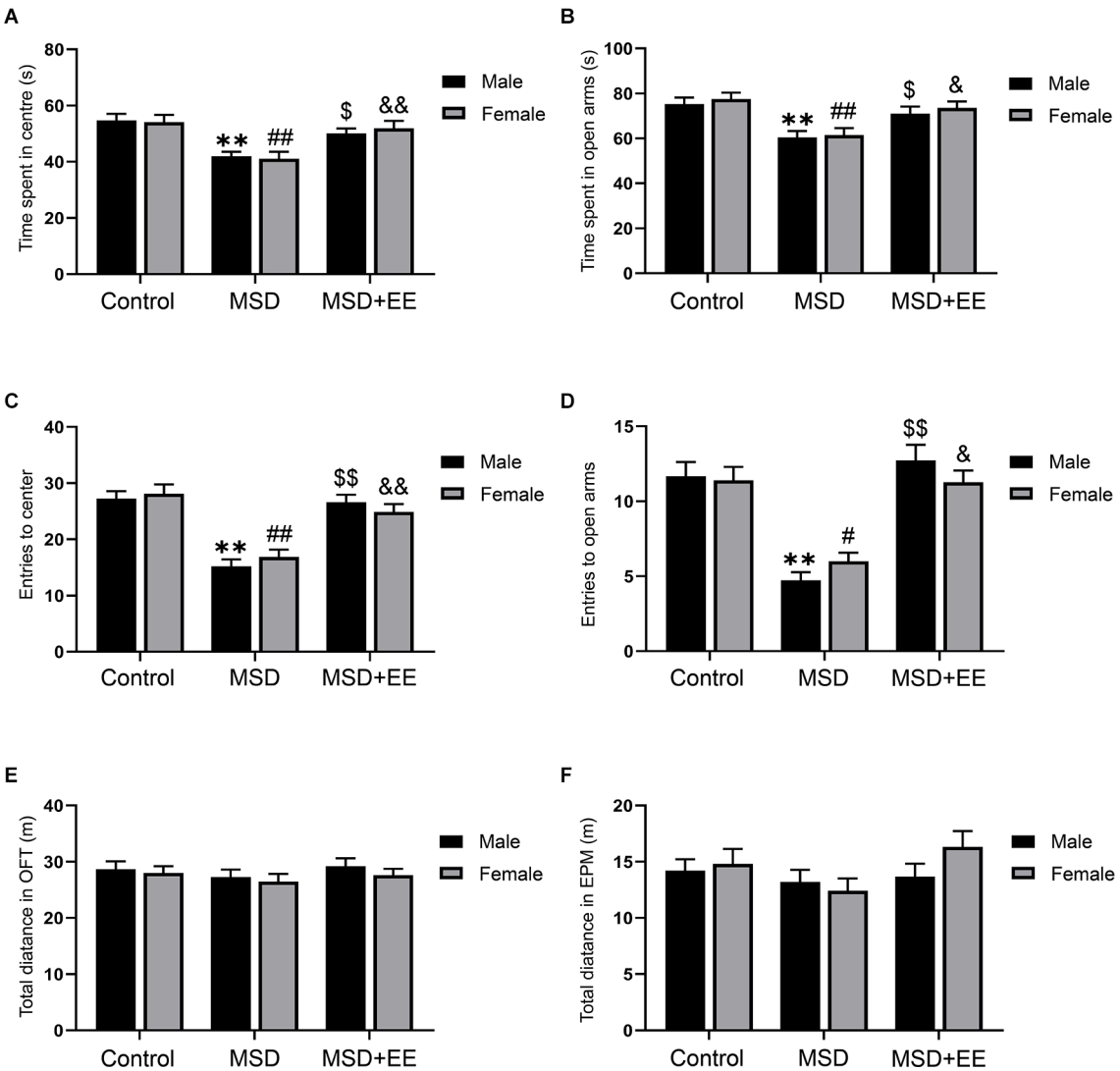

FIGURE 2 | The effect of EE and MSD on anxiety-like behaviors. **(A)** The time spent in the center of the three groups during the open field test. **(B)** The time spent in open arms of the three groups during the elevated plus maze. **(C)** The number of the entries to the center of the open field test. **(D)** The number of the entries to the open arms of the elevated plus maze. **(E)** The total distance of the open field test. **(F)** The total distance of the elevated plus maze. $^{**}P < 0.01$ vs. control male; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control female; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. MSD male; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. MSD female.

Environmental Enrichment Improves Cognitive Impairment Induced by Maternal Sleep Deprivation

The MWM was used to evaluate the effect of EE on MSD-induced cognitive deficits. In the learning phase, a repeated-measures ANOVA revealed no sex differences in escape latency (Control group: $F_{(1,28)} = 0.05$, $P > 0.05$; **Figure 3A**; MSD group: $F_{(1,28)} = 0.14$, $P > 0.05$; **Figure 3B**; MSD+EE group: $F_{(1,28)} = 0.24$, $P > 0.05$; **Figure 3C**) and swimming velocity (Control group: $F_{(1,28)} = 0.98$, $P > 0.05$; **Figure 3D**; MSD group: $F_{(1,28)} = 3.08$, $P > 0.05$; **Figure 3E**; MSD+EE group: $F_{(1,28)} = 1.00$, $P > 0.05$; **Figure 3F**) for each group when the analysis was controlled for treatment. However, a repeated-measures ANOVA for latency from different groups showed significant differences when the analysis was controlled for sex (male group: $F_{(2,42)} = 23.19$, $P < 0.01$; **Figure 3G**; female: $F_{(2,42)} = 16.39$, $P < 0.01$; **Figure 3H**). The *post hoc* analysis revealed that both male and female mice from the MSD group spent more time to find the hidden platform than those in the control group ($Ps < 0.01$), while both male and female mice from the MSD+EE group spent less time to find the hidden platform than those in the MSD group ($Ps < 0.05$). There were no sex differences in swimming velocity within any of the three groups (male group: $F_{(2,42)} = 3.24$, $P = 0.05$; **Figure 3I**; female group: $F_{(2,42)} = 0.38$, $P > 0.05$; **Figure 3J**).

In the memory phase, the two-way ANOVA showed that the time spent in the target quadrant was significantly different between the three groups ($F_{(2,84)} = 34.30$, $P < 0.01$; **Figure 3K**). *Post hoc* comparisons showed the time percent was lowest in the MSD group ($Ps < 0.05$). EE could ameliorate, but not normalize, the time percent of the MSD+EE group when compared to the control group ($P < 0.05$). These results indicated that EE improved MSD-related spatial learning and memory impairment.

Effect of Enriched Environment and Maternal Sleep Deprivation on BDNF and Syt-1 mRNA Levels

A two-way ANOVA showed significant between-group differences in the mRNA levels of BDNF and Syt-1 (BDNF: $F_{(2,42)} = 99.36$, $P < 0.01$; Syt-1: $F_{(2,42)} = 102.82$, $P < 0.01$; **Figures 4A,B**). The *post hoc* analysis revealed that the MSD group had a lower BDNF mRNA level and higher Syt-1 mRNA level than the control group ($Ps < 0.01$). EE increased the BDNF mRNA level and Syt-1 mRNA level, as shown by the MSD+EE group vs. MSD group comparison ($Ps < 0.05$).

Effect of Enriched Environment and Maternal Sleep Deprivation on BDNF and Syt-1 Protein Levels

We further evaluated the protein levels of BDNF and Syt-1 using Western blotting. A two-way ANOVA showed that the protein levels of BDNF and Syt-1 were significantly different between the three groups (BDNF: $F_{(2,30)} = 156.01$, $P < 0.01$; Syt-1: $F_{(2,30)} = 379.71$, $P < 0.01$; **Figures 5A–C**). The *post hoc* analysis revealed that MSD decreased the protein level of BDNF and increased the protein level of Syt-1, as revealed by the MSD group vs. control group comparison ($Ps < 0.01$). EE increased the BDNF protein level and decreased the Syt-1 protein level in mice from the MSD+EE group relative to the MSD group ($Ps < 0.01$).

DISCUSSION

This study investigated the beneficial effects of EE on MSD-induced anxiety-like behavior and spatial cognition dysfunction, as well as synaptic plasticity markers changes in offspring CD-1 mice. Our results indicate that EE could be a useful strategy to alleviate the anxiety-like behaviors and cognition dysfunction caused by MSD. Preservation of cognitive function was associated with an increase in BDNF and a decrease in Syt-1 in the hippocampus.

Insufficient sleep during pregnancy is a public health problem that brings serious mental and psychological problems to offspring, and also increases the financial burden on society (Oyiengo et al., 2014). Therefore, it is interesting and meaningful to use an MSD rodent model to explore the behavioral phenotypes and their underlying biological mechanisms in MSD-induced offspring. There have been conflicting results on the anxiety-like behavior of MSD-induced offspring. One study suggested that Sprague-Dawley rats born to mothers undergoing sleep deprivation during pregnancy displayed anxiety-like behavior during the EPM and novelty-suppressed feeding task (Peng et al., 2016). Another study reported that Wistar rats exposed to MSD during gestation showed a decrease in anxiety-related behavior during the EPM (Radhakrishnan et al., 2015). In the present study, we found that the offspring of MSD CD-1 mice showed anxiety-like behavior, as indicated by decreased time and entries in the center and open arms during OFT and EPM, separately. It may be that offspring from different strains have different tolerances to MSD. Furthermore, the MWM results suggested that MSD impaired hippocampus-dependent learning and memory, which has been validated in numerous experiments (Zhao et al., 2014; Peng et al., 2016). Moreover, we found that EE reversed the levels of MSD-induced anxiety, and alleviated—yet did not normalize—MSD-induced cognitive dysfunction. The hippocampus is an important brain region for learning and memory, and its morphology and synaptic function are more susceptible to stress and more difficult to repair than emotion-related regions (Guan et al., 2004; Ruskin et al., 2004). To the best of our knowledge, our experiment is the first to demonstrate the beneficial effect of EE on MSD-associated anxiety-like behavior and cognitive impairment.

BDNF is widely expressed in the hippocampus and has potent effects on cognitive function (Autry and Monteggia, 2012). *In vitro* study, BDNF can promote the survival, proliferation, and differentiation of neural stem cells (Chen et al., 2013; Hachem et al., 2015). *In vivo* study, the heterozygous (*BDNF*+/-) mice with knockout of *BDNF* showed a decrease in cell proliferation and survival in the dentate gyrus of the hippocampus (Lee et al., 2002). The (*BDNF*^{2lox}/*BDNF*^{2lox}/*CaMKII-cre*) mice with conditional knockout of *BDNF* in mature neurons exhibited an impaired dendritic development without affecting cell proliferation and differentiation (Choi et al., 2009). The inconsistent results regarding the effects of BDNF are due

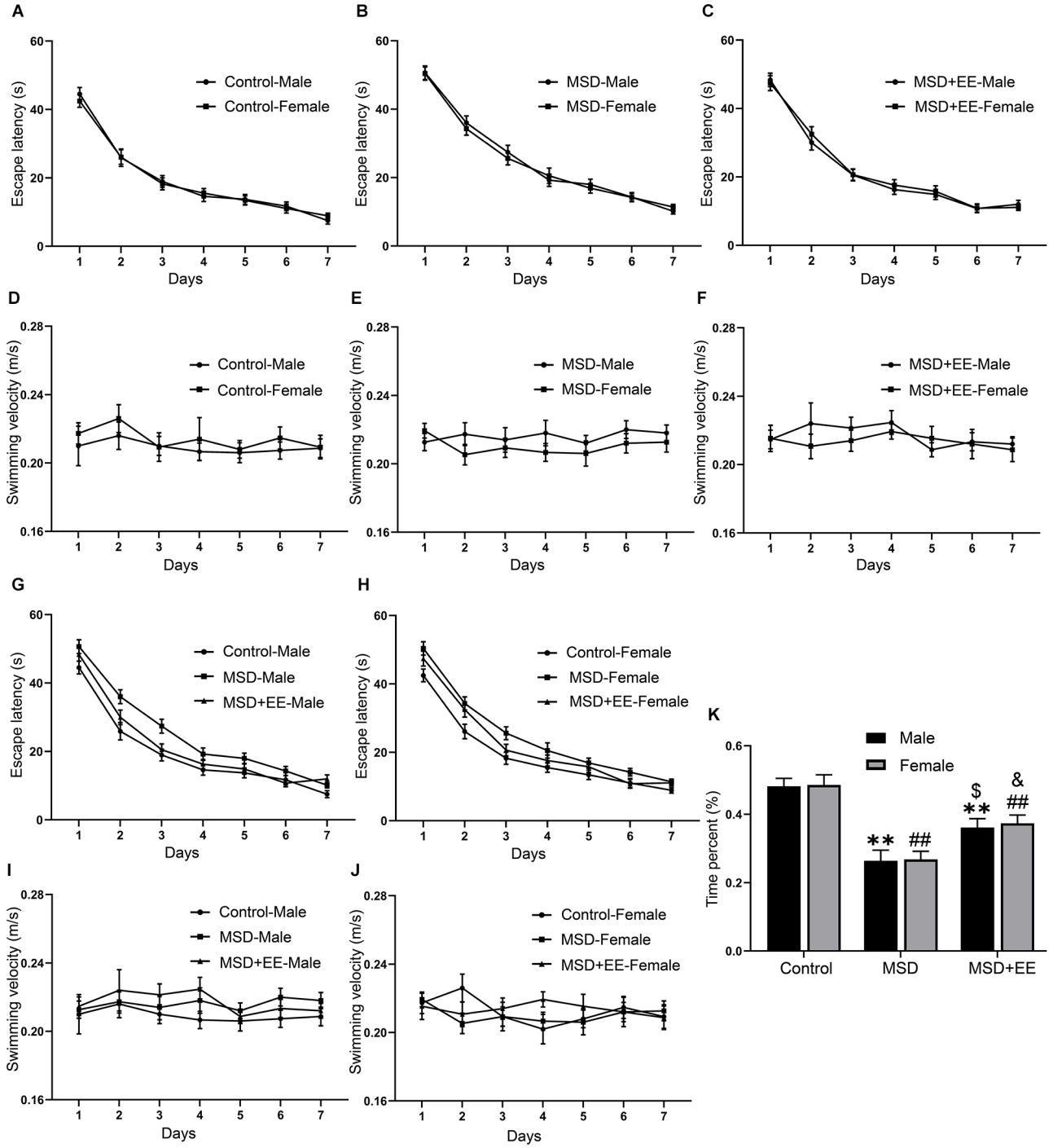

FIGURE 3 | EE attenuated the decline in spatial learning and memory ability of MSD-induced mice. In the learning phase, the escape latency **(A,B,C,G,H)** and swimming velocity **(D,E,F,I,J)** for each of the seven training days are shown. In the memory phase, the percent time spent in the target quadrant is shown **(K)**. $^{**}P < 0.01$ vs. control male; $^{\#\#}P < 0.01$ vs. control female; $^{\$}P < 0.05$ vs. MSD male; $^{\&}P < 0.05$ vs. MSD female.

to differences in experimental methods and gene knockout techniques. A previous study had demonstrated that the C57BL/6 mice exposed to isoflurane during aging exhibited cognitive deficits, which were accompanied by inhibition of the BDNF pathway and downregulation of synaptic plasticity markers in the hippocampus (Wu et al., 2016). Similarly, our results showed that MSD resulted in impaired cognitive function accompanied by a downregulation of BDNF. Furthermore,

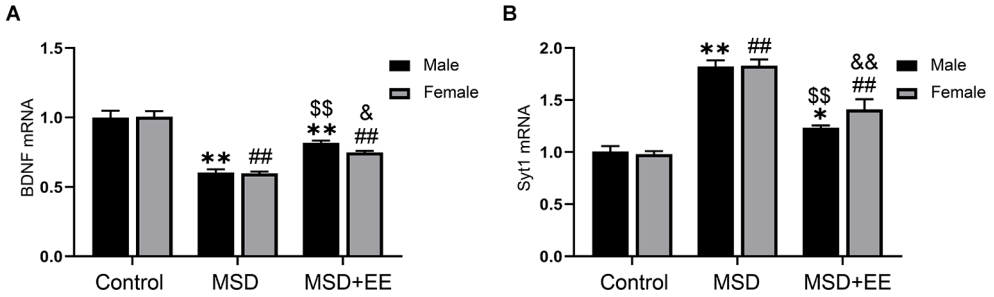

FIGURE 4 | Relative mRNA levels of BDNF and Syt-1 in the hippocampus. **(A)** The mRNA level of BDNF in the hippocampus of the three groups. **(B)** The mRNA level of Syt-1 in the hippocampus of the three groups. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control male; $^{\#\#}P < 0.01$ vs. control female; $^{\$\$}P < 0.01$ vs. MSD male; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. MSD female.

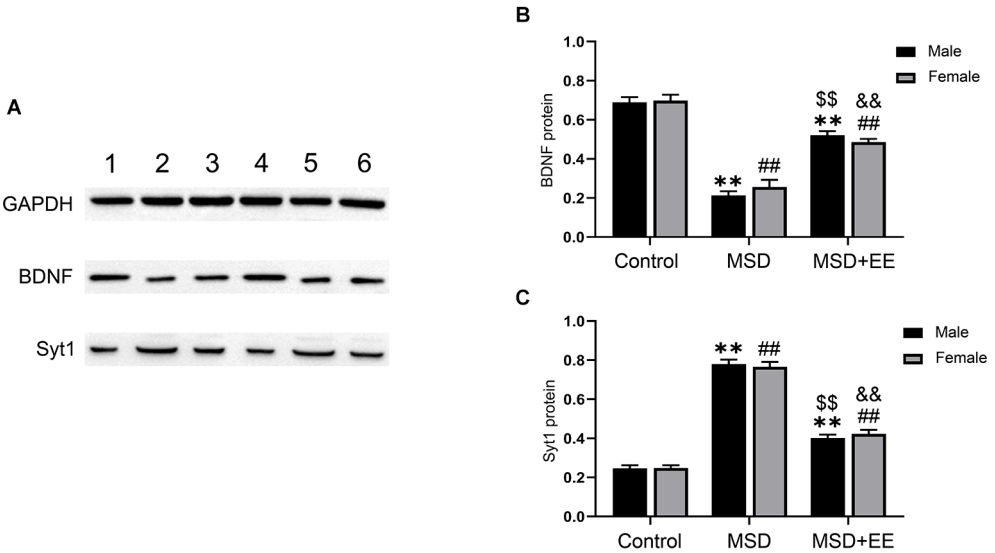

FIGURE 5 | Hippocampal protein levels of BDNF and Syt-1. **(A)** Western blot: band 1: Control-male; band 2: MSD-male; band 3: MSD+EE-male; band 4: Control-female; band 5: MSD-female; band 6: MSD+EE-female. **(B)** The protein level of BDNF in the hippocampus of the three groups. **(C)** The protein level of Syt-1 in the hippocampus of the three groups. $^{**}P < 0.01$ vs. control male; $^{\#\#}P < 0.01$ vs. control female; $^{\$\$}P < 0.01$ vs. MSD male; $^{\&\&}P < 0.01$ vs. MSD female.

a previous study demonstrated that adolescent enriched environment could alleviate sleep deprivation-associated cognition dysfunction by restoring BDNF expression levels in male Wistar rats (Ghaheri et al., 2022). Our Western blotting and real-time fluorescence quantitative polymerase chain reaction results have suggested that EE increases BDNF mRNA and protein levels in the hippocampus of MSD mice. A previous study showed that long-term treatment of hippocampal slice cultures with BDNF increased the number of docked vesicles at hippocampal CA1 synapses and increased the protein levels of synaptotagmin, synaptophysin, and synaptobrevin (Tartaglia et al., 2001). The interaction between the synaptic vesicle-associated proteins and BDNF might trigger the imbalance of synaptic plasticity that occurs in cognitive impairment. The downregulation of Syt-1 has been implicated in chronic brain hypoperfusion-associated presynaptic plasticity dysfunction and treadmill exercise training improved hippocampus-associated learning and memory by upregulation of Syt-1, which indicated that the expression level of Syt-1 was positive with cognitive function (Liu et al., 2009; Yan et al., 2020). Surprisingly, our results showed that MSD-induced cognitive impairment was associated with increased Syt-1 expression. The results are in accordance with a previous report that upregulation of Syt-1 in the hippocampus has been found to cause neuron damage associated with prenatal stress- and hypothyroidism-induced cognitive impairment (Vara et al., 2002; Jia et al., 2010). Moreover, EE could improve MSD associated-cognitive deficits by reducing Syt-1 expression, which was consistent with our previous study that exposure to an EE from adolescence improved age-associated cognitive decline by downregulating the expression of Syt-1 (Zhang et al., 2022). The contradictory results regarding the relationship between Syt-1 expression level and cognitive function could be a result of differences in the

specific mechanisms leading to cognitive impairment in different pathological models.

Importantly, the mechanisms underlying MSD-related cognitive impairment involve not only changes in synaptic markers but also alterations in inflammation and the hypothalamic-pituitary-adrenal axis (Zhao et al., 2014; Ehichioya et al., 2022). Previous studies suggested that EE could improve cognitive dysfunction by altering pro-inflammatory cytokines and hyperactivity of the hypothalamic-pituitary-adrenal axis (Delanogare et al., 2020; Keymoradzadeh et al., 2020). The effects of EE on inflammatory and hypothalamic-pituitary-adrenal markers should be further explored in MSD models. Moreover, we found that EE attenuated MSD-associated impaired cognition, but did not fully reverse it. Further studies could examine the effect of drugs and other non-drug treatments, such as exercise, on MWM performance in MSD-induced mice (Liu et al., 2009).

Our study has limitations. First, we only used Western blotting and real-time fluorescence quantitative polymerase chain reaction to evaluate the expression levels of BDNF and Syt-1, and did not use immunohistochemistry to quantitatively analyze the effect of EE on the expression levels of BDNF and Syt-1 in different subregions of the hippocampus. Second, we did not further assess hippocampal synaptic plasticity resulting from changes in synaptic plasticity markers by patch-clamp technique. Third, we did not use RNA interference technology to reduce BDNF and increase Syt-1 to verify the targets of EE.

CONCLUSION

Our study demonstrated that MSD-induced offspring exhibited anxiety-like behavior, cognitive impairment, and BDNF and Syt-1 expression. Notably, sleep deprivation during pregnancy induced randomly without analyzing the circadian rhythm and their activity pattern. Access to EE alleviated anxiety-like behavior and cognitive impairment in offspring from the MSD group. The improved cognitive function can be partially explained by an increase in BDNF and a decrease in Syt-1 in the hippocampus. Thus, EE treatment may have utility for the prevention of the development of anxiety-like behaviors and recovery from cognitive deficits following sleep deprivation during pregnancy.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

The animal study was reviewed and approved and all animal experiments were carried out in compliance with the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (No. LLSC20190710).

AUTHOR CONTRIBUTIONS

Y-MZ and Y-ZC designed the study, performed behavioral tests, and drafted the manuscript. Y-TW and R-MW were responsible for western blotting and real-time fluorescence quantitative polymerase chain reaction. Y-JG and X-YK analyzed the data and made graph. X-YL revised the manuscript and were responsible for the completeness and accuracy of the data. All authors contributed to the article and approved the submitted version.

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Heterozygous Deletion of Epilepsy Gene *KCNQ2* Has Negligible Effects on Learning and Memory

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Neuronal K_V7/Potassium Voltage-Gated Channel Subfamily Q (KCNQ) potassium channels underlie M-current that potently suppresses repetitive and burst firing of action potentials (APs). They are mostly heterotetramers of $K_V7.2$ and $K_V7.3$ subunits in the hippocampus and cortex, the brain regions important for cognition and behavior. Underscoring their critical roles in inhibiting neuronal excitability, autosomal dominantly inherited mutations in Potassium Voltage-Gated Channel Subfamily Q Member 2 (*KCNQ2*) and Potassium Voltage-Gated Channel Subfamily Q Member 3 (*KCNQ3*) genes are associated with benign familial neonatal epilepsy (BFNE) in which most seizures spontaneously remit within months without cognitive deficits. *De novo* mutations in *KCNQ2* also cause epileptic encephalopathy (EE), which is characterized by persistent seizures that are often drug refractory, neurodevelopmental delay, and intellectual disability. Heterozygous expression of EE variants of *KCNQ2* is recently shown to induce spontaneous seizures and cognitive deficit in mice, although it is unclear whether this cognitive deficit is caused directly by K_V7 disruption or by persistent seizures in the developing brain as a consequence of K_V7 disruption. In this study, we examined the role of K_V7 channels in learning and memory by behavioral phenotyping of the *KCNQ2*$^{+/-}$ mice, which lack a single copy of *KCNQ2* but dos not display spontaneous seizures. We found that both *KCNQ2*$^{+/-}$ and wild-type (WT) mice showed comparable nociception in the tail-flick assay and fear-induced learning and memory during a passive inhibitory avoidance (IA) test and contextual fear conditioning (CFC). Both genotypes displayed similar object location and recognition memory. These findings together provide evidence that heterozygous loss of *KCNQ2* has minimal effects on learning or memory in mice in the absence of spontaneous seizures.

Keywords: *KCNQ2*, K_V7 channel, learning, memory, nociception

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INTRODUCTION

Voltage-gated potassium (K^+) channel subfamily Q (K_V7/Potassium Voltage-Gated Channel Subfamily Q [KCNQ]) is a critical regulator of neuronal excitability (Greene and Hoshi, 2017; Baculis et al., 2020). In the central nervous system, K_V7 channels are mostly heterotetramers of $K_V7.2$ and $K_V7.3$ subunits and to a lesser extent, heterotetrameric $K_V7.3$ and $K_V7.5$ channels and

homomeric $K_v7.2$ channels (Baculis et al., 2020). $K_v7.2$ and $K_v7.3$ show overlapping expression in the hippocampus and cortex, the brain regions critical for cognition and behavior (Cooper et al., 2001; Pan et al., 2006), and are highly concentrated at the axonal plasma membrane that include the initial segment when compared to the dendritic plasma membrane (Chung et al., 2006; Pan et al., 2006). Upon membrane depolarization, K_v7 channels mediate slowly activating and non-inactivating outward K^+ current called M-current (I_M) (Brown and Passmore, 2009) that potently suppresses action potential (AP) firing rate and burst firing, hyperpolarizes resting membrane potential, and regulates spike threshold and after hyperpolarization (Aiken et al., 1995; Gu et al., 2005; Shah et al., 2008; Greene and Hoshi, 2017; Baculis et al., 2020). Additionally, K_v7 channels in hippocampal pyramidal neurons produce intrinsic theta resonance called M-resonance at depolarized subthreshold potentials (Peters et al., 2005).

Underscoring the critical roles of K_v7 channels in inhibiting neuronal excitability (Greene and Hoshi, 2017; Baculis et al., 2020), the agonist retigabine reduces seizures in animal models and humans (Miceli et al., 2008), whereas dominant mutations in either Potassium Voltage-Gated Channel Subfamily Q Member 2 (*KCNQ2*) or Potassium Voltage-Gated Channel Subfamily Q Member 3 (*KCNQ3*) genes cause neonatal epilepsy that includes benign familial neonatal epilepsy (BFNE) and epileptic encephalopathy (EE) (www.rikee.org, www.ncbi.nlm.nih.gov/clinvar/). In most patients with BFNE, neonatal seizures fully abate within weeks to months after birth (Miceli et al., 2011; Soldovieri et al., 2011). In contrast, patients with EE display early-onset intractable seizures, which are often drug resistant (Weckhuysen et al., 2012; Nappi et al., 2020). Most BFNE and EE mutations impair voltage-dependent activation, phosphatidylinositol bisphosphate (PIP_2) sensitivity, and/or axonal enrichment of K_v7 channels (Miceli et al., 2011; Soldovieri et al., 2011; Weckhuysen et al., 2012; Cavaretta et al., 2014; Kim et al., 2018; Nappi et al., 2020; Zhang et al., 2020). Furthermore, heterozygous knock-in mice for BFNE mutants $K_v7.2^{Y284C}$, $K_v7.2^{A306T}$, or $K_v7.3^{G311V}$ show heightened seizure susceptibility (Singh et al., 1998, 2008), whereas heterozygous expression of the EE variant $K_v7.2^{T274M}$ or $K_v7.2^{M547V}$ induces spontaneous seizures and early mortality in mice (Milh et al., 2020; Kim et al., 2021), suggesting that K_v7 dysregulation contributes to BFNE and EE.

In addition to seizures, patients with EE develop neurodevelopmental delay and intellectual disability (Zhang et al., 2020) and *de novo* dominant mutations in *KCNQ2* and *KCNQ3* genes have recently been associated with neurodevelopmental delay without seizures (Coe et al., 2019), suggesting the possible role of K_v7 channels in learning and memory (Baculis et al., 2020). Indeed, K_v7 channel antagonists, linopirdine and XE991, enhance fear-motivated avoidance learning and object recognition task performance in rodents in the mouse model of dementia (Cook et al., 1990; Fontan-Lozano et al., 2011). Stimulation of Gq-coupled muscarinic acetylcholine receptors (mAChRs) and subsequent inhibition of I_M (Brown and Passmore, 2009) in the prefrontal cortex (PFC) prevents the decline in working memory induced by cholinergic depletion in aging primates (Galvin et al., 2020). In contrast to these beneficial effects of acute pharmacological inhibition of K_v7 channels on cognition, genetic knock-down or ablation of a single Drosophila Potassium Voltage-Gated Channel Subfamily Q Member 1 (*dKCNQ*) gene in *Drosophila* is shown to induce ethanol hyperexcitability (Cavaliere et al., 2012) and impair both short-term memory and long-term memory, respectively (Cavaliere et al., 2013). In mice, genetic suppression of I_M by overexpression of the dominant negative mutant $K_v7.2^{G279S}$ or EE mutant $K_v7.2^{T274M}$ or $K_v7.2^{M547V}$ in the developing brain induces spontaneous seizures and impairs learning and memory (Peters et al., 2005; Milh et al., 2020; Kim et al., 2021). However, it is difficult to tease out whether cognitive deficits in the genetic mouse models arise directly from the reduction of I_M or from indirect consequences of persistent seizures induced by I_M suppression.

Here, we investigated the role of $K_v7.2$-containing channels in learning and memory by behavioral phenotyping of the $KCNQ2^{+/-}$ mice that were heterozygous null for *KCNQ2* but show a normal level of *KCNQ3* transcript (Tzingounis and Nicoll, 2008). Consistently, $KCNQ2^{+/-}$ mice display reduced $K_v7.2$ but not $K_v7.3$ expression in their hippocampi when compared to the wild-type (WT) mice (Kim et al., 2019). Although I_M in $KCNQ2^{+/-}$ mice as compared to WT mice has not been reported, the $KCNQ2^{+/-}$ dentate granule cells display a 50% decrease in the amplitudes of medium and slow after hyperpolarization currents (Tzingounis and Nicoll, 2008), suggesting the contribution of $K_v7.2$-containing channels. $KCNQ2^{+/-}$ mice were chosen because they are viable and do not show spontaneous seizures (Watanabe et al., 2000; Tzingounis and Nicoll, 2008) in sharp contrast to homozygous *KCNQ2* knock-out mice that are perinatal lethal (Watanabe et al., 2000; Tzingounis and Nicoll, 2008) and conditional homozygous forebrain knock-out mice of *KCNQ2*, which display spontaneous seizures and early mortality by weaning age (Soh et al., 2014). In addition, $KCNQ2^{+/-}$ mice and WT $KCNQ2^{+/+}$ mice show comparable levels of locomotor activity and motor coordination (Kim et al., 2019), making $KCNQ2^{+/-}$ mice a suitable model to study the effect of $K_v7.2$ haploinsufficiency on learning and memory in the absence of spontaneous seizures. In this study, we discovered that fear-motivated learning and memory, spatial memory, and object recognition memory were unaffected by the heterozygous loss of *KCNQ2*. Interestingly, $KCNQ2^{+/-}$ mice display a longer total time to reach the criterion than the WT mice during the acquisition phase of the inhibitory avoidance (IA) task, suggesting a possible specific deficit in decision-making and/or fear perception.

MATERIALS AND METHODS

Experimental Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana Champaign. $KCNQ2^{+/-}$ mice on the C57BL/6J background have been obtained from the Jackson Laboratory [$Kcnq2^{tm1Dgen}/Kcnq2^{+}$, Stock Number: 005830 (Tzingounis and Nicoll, 2008)]. $KCNQ2^{+/-}$ mice were bred against C57BL/6J

mice and housed on a normal 12:12 light:dark cycle (lights on at 6 a.m. and lights off at 6 p.m.) with food and water available *ad libitum*. At weaning, the littermates of the same sex were group-housed with up to 5 mice per cage and were genotyped as described (Kim et al., 2019).

Behavior Studies

A total of 66 male mice were used ($KCNQ2^{+/+}$ $n = 37$; $KCNQ2^{+/-}$ $n = 29$) and a total of 78 female mice were used ($KCNQ2^{+/+} = 36$; $KCNQ2^{+/-}$ $n = 42$) for behavior tests at 4–6 months of age. Experimenters were blind to the mouse genotype. The motor coordination of $KCNQ2^{+/-}$ mice has been previously reported to be similar to their WT littermates (Kim et al., 2019). Because $KCNQ2^{+/-}$ mice display hyperactivity in the light but not in dark phase (Kim et al., 2019), both genotypes were tested for the passive IA test, contextual fear conditioning (CFC), object location task (OLT), and novel object recognition task (NORT) during the dark phase in a separate "behavior" room from the rest of the colony. Specifically, these tasks were performed 2 h after the start of the dark phase. To eliminate possible pain-induced effects on future behaviors, OLT and NORT were followed by passive IA a minimum of 10 days later, and separate cohorts were used for CFC and tail-flick assay. Furthermore, we used both male and female mice since sex differences in social dominance and compulsive behavior were previously reported in $KCNQ2^{+/-}$ mice (Kim et al., 2019).

Passive IA, OLT, and NORT were performed in a behavior room maintained on a "reverse" light:dark cycle (lights off at 10 a.m. and lights on at 10 p.m.) after the mice were single housed and habituated to both this cycle and handling for 2 weeks. Due to a conflict with the availability of the behavior room maintained on a reverse light:dark schedule, the mice for CFC were subjected to this task during the dark phase in a different behavior room maintained in a normal 12:12 light:dark cycle after habituation to the handling for 2 weeks. Compared to the dark phase, mice display higher pain sensitivity in the light phase (Kavaliers and Hirst, 1983). Therefore, the tail-flick assay was performed on both genotypes in the light phase, which may better reveal the genotype-specific difference in nociception. Hyperactivity of $KCNQ2^{+/-}$ mice in the light phase as compared to the WT mice (Kim et al., 2019) is expected to have a negligible effect on the thermal nociception examined by the tail-flick assay, which does not require the locomotion of mice. All behavior apparatus was cleaned with 70% ethanol between each mouse.

The passive IA was performed in a dedicated behavior room as described (Hamilton et al., 2017). On Day 1 (training), the mouse was placed in a two-chamber GEMINI Avoidance System (San Diego Instruments) and the start button was immediately pressed to turn on the LED light in the chamber containing the mouse and simultaneously raise the gate separating the two chambers. When the mouse crossed into the dark chamber, the gate was closed and the mouse received a mild foot shock (0.5 mA) for 4 s (s). After 10 s, the LED house light was turned back on in the chamber containing the mouse and the gate was opened, allowing the mouse to cross to the dark chamber. This procedure was repeated until the mouse reached the criterion by remaining in the lit chamber for 120 s or until 50 attempts had been made without meeting the criterion. The latency to cross per trial and the number of crosses to reach the criterion during the training period were recorded. At 24 and 48 h after training, the mouse was placed again in the avoidance system without foot shocks, and the latency to cross to the dark chamber was recorded up to 300 s. If the mouse had not crossed into the dark chamber by 300 s, the trial was marked as a "no cross" and latency was recorded as 300 s.

The CFC task was performed as described (Kohman et al., 2012) in the chamber containing a metal grate that spanned the bottom of the box and administered a foot shock. On each day of experimentation, the mice were first separated into a single housing for 4–5 h in a separate behavior room and then tested starting at 2 h into the dark phase under video recording. On Day 1, the mouse was placed in the chamber for habituation for 180 s. At 24 h after habituation on Day 2 (training), the mouse was placed in the same chamber for 180 s during which a mild foot shock (0.5 mA, 2 s) was delivered at 120 and 150 s. At 24 h after training on Day 3 (testing), the mouse was placed in the same chamber for 180 s without foot shocks. The total freezing time was recorded on each day. Freezing was defined as a total lack of movement outside of breathing. At the end of experiments in each day, the mice were returned to their original group housing. The percentage (%) of total time spent freezing was calculated as $100\left(\frac{freezing\ time\ (s)}{180\ s}\right)$.

The OLT and NORT were performed with video recording as described (Denninger et al., 2018). On Day 1, the mouse was placed in a designated release corner of the empty test chamber and allowed to explore for three separate 6-min intervals. At 24 h after habituation on Day 2, the mouse was placed back into the chamber and allowed to explore the objects for 10 min in 3 different test phases. In phase 1 (training), the chamber contained two distinct objects secured at 6 × 6 cm^2 from its two corners. In phase 2 (OLT), one of the objects was moved to a new location at the opposite corner. In phase 3 (NORT), the object that was previously moved in OLT was replaced with a novel object. After each phase, the mouse was placed in their holding cage for 20 min. TopScan (CleverSys) was used to track the movement of a mouse in the chamber and record the duration of its investigation of an object, which is defined as when its head was oriented toward the object within 1 cm or when its nose was touching the object. The discrimination index (DI) was calculated as $100\left(\frac{time\ sniffing\ novel\ object\ or\ location - time\ sniffing\ familiar\ object\ or\ location}{time\ sniffing\ novel + time\ sniffing\ familiar\ object\ or\ location}\right)$. The mice that have DI $> +0.2$ or < -0.2 during training were considered to have a significant location and/or object bias during training and were excluded from statistical analysis as previously described (Vogel-Ciernia and Wood, 2014).

The tail-flick assay was performed as described (Schildhaus et al., 2014). Two 500 ml beakers were filled with 450 ml of distilled water and placed on stir plates with induction heaters to maintain even water temperatures of 36 and 51°C. During the test, the stir plates were turned off and the tail of the mouse was lowered 3 cm into the 36°C bath for 30 s or until it started to flick rapidly. The latency until the tail-flick was recorded. The tail was

dried and returned to room temperature. The same procedure was then repeated for the 51°C bath.

Statistical Analysis

All analyses are reported as mean ± SEM. The n values indicate the number of mice. Origin Pro 9.5 (Origin Lab) was used to perform statistical analyses. When the data were separated by sex from passive IA and CFC, tail-flick tests (**Supplementary Figures S1**, **S2**) were analyzed using the 2-way ANOVA with genotype as one factor and sex as the other, there were no significant effects of sex and no interactions between sex and genotype (**Supplementary Table S1**). Therefore, the data for male mice and female mice were combined and analyzed by a two-tailed student's *t*-test for comparing 2 groups and a *post-hoc* Tukey test for comparing >2 groups. *A priori* value (p) < 0.05 was used to establish statistical significance.

RESULTS

Heterozygous Loss of *KCNQ2* Has Minimal Effects on IA and Does Not Affect Contextual Fear-Induced Learning or Memory

To test the role of $K_v7.2$-containing channels in fear-motivated learning and memory in mice, adult *KCNQ2*$^{+/-}$ mice, and their WT littermates (*KCNQ2*$^{+/+}$) at 4–6 months of age were subjected to the passive IA and CFC. These tasks are dependent on the hippocampus, amygdala, and PFC (Kohman et al., 2012; Hamilton et al., 2017). In the passive IA, which exploits a rodent's natural preference for the dark environment (Hamilton et al., 2017), the criterion for fear-motivated learning is established during training when the mouse remains in the lit chamber for 120 s rather than entering the dark chamber where it receives a foot shock (**Figure 1A**). We found that *KCNQ2*$^{+/-}$ mice took a longer time to cross in the fifth trial and displayed a longer total time to reach the criterion during training as compared to *KCNQ2*$^{+/+}$ mice (**Figures 1B,C**, **Supplementary Figure S1**). These results together suggest that *KCNQ2*$^{+/-}$ mice were more hesitant to cross into the dark chamber where they previously received a shock. However, this did not translate into better learning of the task because both genotypes displayed a similar number of crosses to reach the criterion (**Figure 1D**, **Supplementary Figure S1**). Both genotypes also showed comparable latency to enter the dark chamber at 1–2 days after training (**Figure 1E**, **Supplementary Figure S1**), indicating that fear-induced memory on the IA task was unaffected by heterozygous loss of *KCNQ2*.

To further investigate the role of $K_v7.2$-containing channels in fear-induced memory, CFC was performed (Kohman et al., 2012). This task tests the ability of a mouse to remember and associate the CFC chamber (context) with the foot shocks (aversive stimuli) (**Figure 2A**). During habituation in the CFC chamber without foot shocks, both *KCNQ2*$^{+/+}$ and *KCNQ2*$^{+/-}$ mice displayed minimal freezing (**Figure 2B**, **Supplementary Figure S2A**). At 1-day post-foot shocks, the freezing response duration was significantly increased in both genotypes to a similar extent (**Figure 2B**, **Supplementary Figure S2B**), indicating that heterozygous loss of *KCNQ2* does not affect contextual fear memory.

Heterozygous Loss of *KCNQ2* Does Not Affect Thermal Pain Sensitivity in Mice

A subtle difference in the total time to reach the criterion in passive IA could be caused by the difference in nociception between *KCNQ2*$^{+/-}$ and the WT mice, since functional K_v7 channels exist in dorsal root ganglia neurons (Rose et al., 2011). To test this, we performed the tail-flick assay (Schildhaus et al., 2014) in which the latency to the tail-flick was measured upon incubating the mouse tail in the 51°C water bath as compared to the control in 36°C water bath. We found that both *KCNQ2*$^{+/+}$ and *KCNQ2*$^{+/-}$ mice displayed similar latency to the tail-flick in a hot water bath (**Figure 2C**, **Supplementary Figure S2C**), indicating no significant genotype difference in thermal pain tolerance.

Heterozygous Loss of *KCNQ2* Does Not Affect Object Location and Recognition Memory in Male Mice

To test if heterozygous loss of *KCNQ2* affects memory that is not induced by fear, we next performed the OLT, which evaluates hippocampus-dependent spatial memory, and the NORT, which tests non-spatial memory of object identity (Vogel-Ciernia and Wood, 2014; Denninger et al., 2018). In these tasks, a mouse is first habituated to an empty test chamber. The next day, the mouse explores two distinct objects in the same chamber for 10 min and then is removed for 20 min (training, **Figure 3A**). The mouse returns to the same chamber where one of the objects was moved and explores for 10 min (OLT, **Figure 3A**). After a 20-min break, the mouse returns to the same chamber where one of the objects is replaced with a novel object and explores for 10 min (NORT, **Figure 3A**). After removing mice that showed a significant bias for one object over the other during training as indicated by $0.2 < DI < -0.2$ (see **Supplementary Figure S3B** for percentages of mice that met this criterion), the 2-way ANOVA for both OLT and NORT showed a significant effect of sex and interaction between genotype and sex (**Supplementary Table S1**). The criterion of 0.2 was chosen as the threshold for object bias during training based on Vogel-Ciernia and Wood (2014) since typical DIs for short- and long-term memory range from 0.25 to 0.45 (Vogel-Ciernia and Wood, 2014).

Inspection of the graphs showed that the male groups displayed significant object location and novel object recognition (**Figure 3B**). A small portion of male mice of both genotypes did not perform well as compared to the training phase (**Supplementary Figure S3C**). Since both genotypes display similar motor coordination and travel comparable distance in the open field arena in the dark phase (Kim et al., 2019) and had been habituated for 2 weeks to the handling and reverse light:dark cycle, we speculate that the unexpected environmental stress factors (the noise from the building or a cage change by

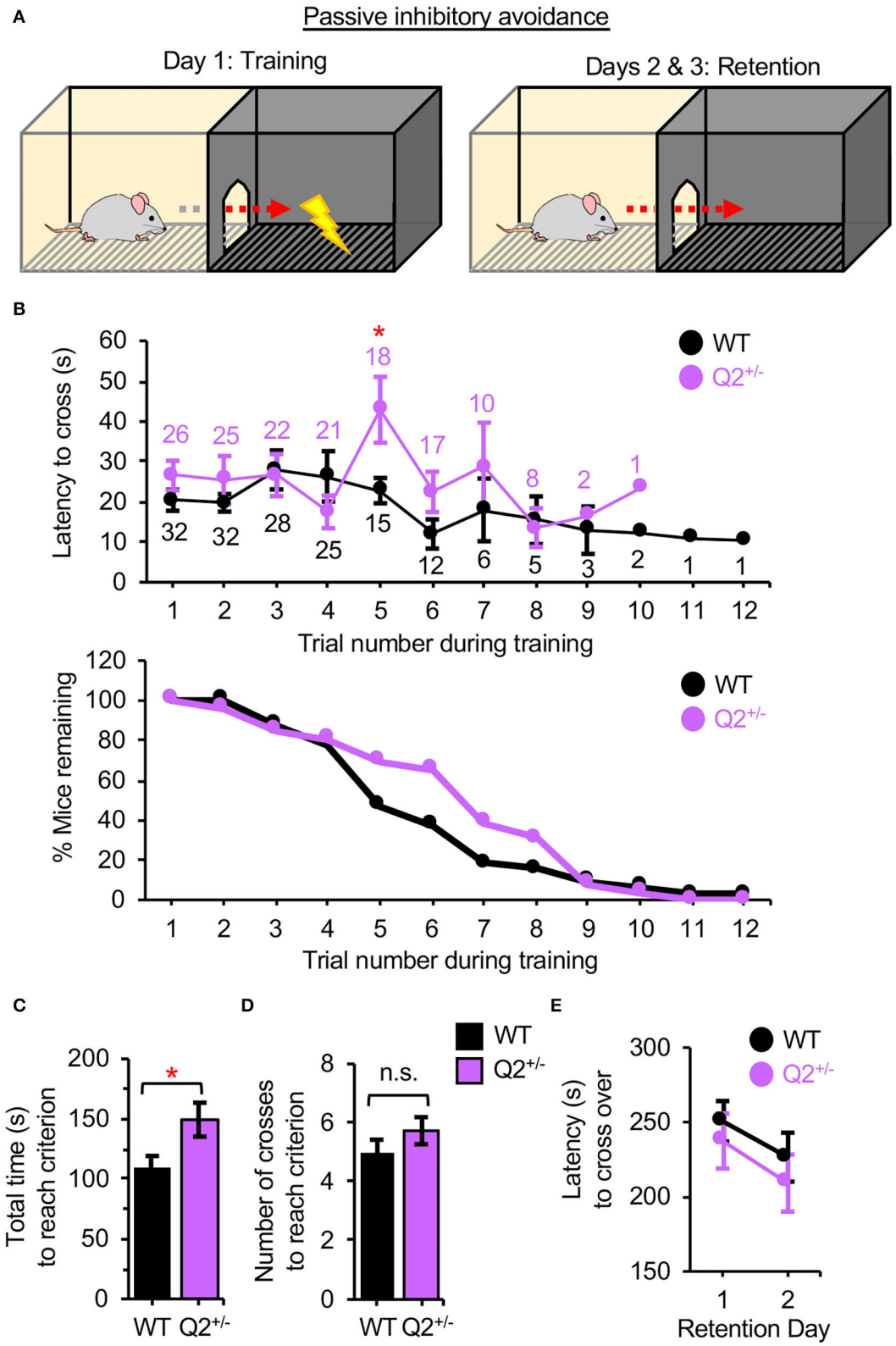

FIGURE 1 | Heterozygous loss of Potassium Voltage-Gated Channel Subfamily Q Member 2 (*KCNQ2*) increases the total time to reach criterion during training on an inhibitory avoidance (IA) task but does not affect memory in mice. *KCNQ2*$^{+/+}$ (wild-type; WT) mice and *KCNQ2*$^{+/-}$ (Q2$^{+/-}$) mice at age 4–6 months were subjected to passive IA test. **(A)** The design of the passive IA test. On the training day, the criterion for fear-motivated learning is established when the mouse remains in the lit

(Continued)

FIGURE 1 | chamber for 120 s rather than entering the dark chamber where it receives a foot shock (0.5 mA, 4 s). At 1 and 2 days after training (retention days), the trained mouse is placed in the lit chamber and fear-induced memory is tested by recording the latency to cross into the dark chamber for a maximum of 300 s. **(B)** The latency to cross into the dark chamber in each trial during training (top graph). The number of WT mice (black) and that of $Q2^{+/-}$ mice (purple) that remained in each trial is also shown. The percentage (%) of mice remaining in each trial during training (bottom graph). **(C)** The total time to reach the criterion during the training was calculated by adding the latency per trial except for the final 120 s when the mouse remained in the lit chamber without crossing. **(D)** The number of crosses during the training. **(E)** The latency to cross into the dark chamber during retention days. Number of mice used: WT ($n = 31$ that includes 14 male mice and 17 female mice), $Q2^{+/-}$ ($n = 26$ that includes 11 male mice and 15 female mice). Data represent the mean ± SEM. Two-tailed student's *t*-test results are shown ($^*p < 0.05$). The individual data points are shown in **Supplementary Figure S1**.

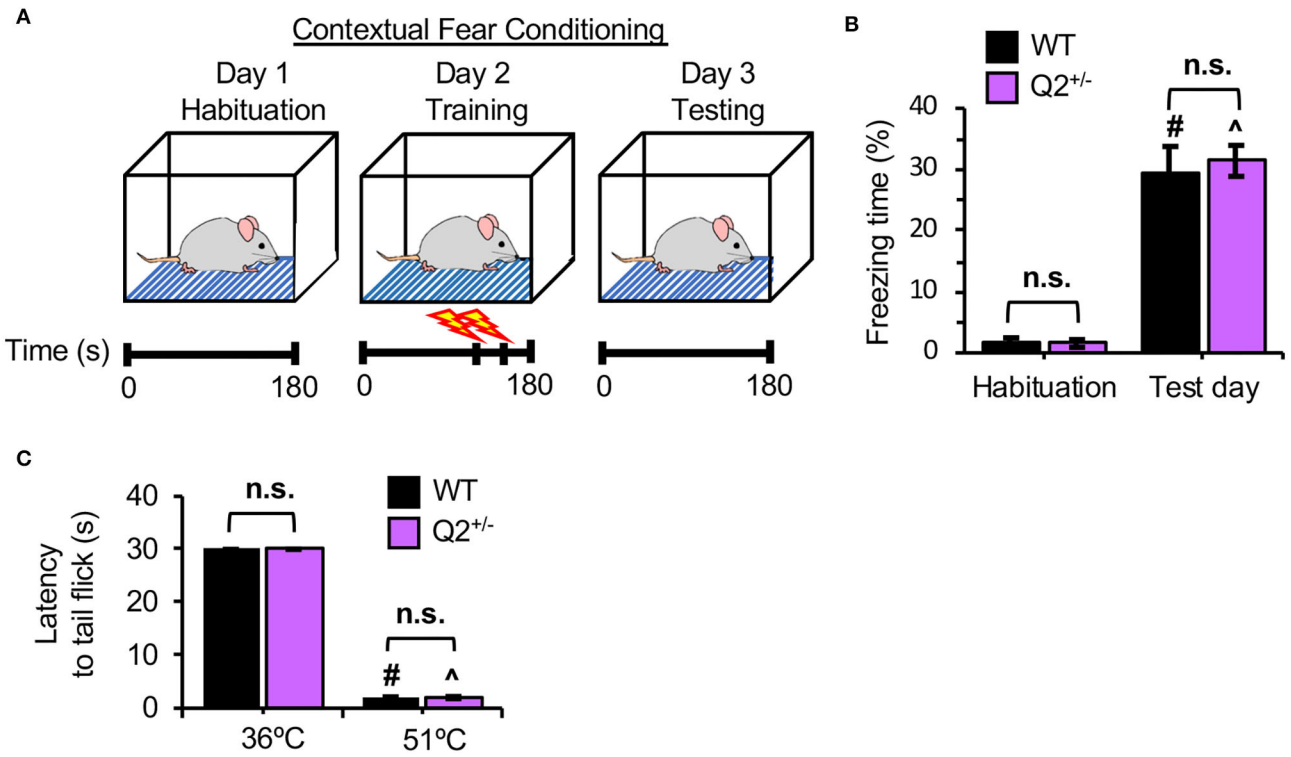

FIGURE 2 | Heterozygous loss of Potassium Voltage-Gated Channel Subfamily Q Member 2 (*KCNQ2*) in mice does not affect fear memory retention and thermal pain threshold. Separate cohorts of $KCNQ2^{+/+}$ (wild-type; WT) mice and $KCNQ2^{+/-}$ ($Q2^{+/-}$) mice at age 4–6 months were subjected to contextual fear conditioning (CFC) test and tail-flick assay. **(A)** The design of the CFC test. A mouse is first habituated in a CFC chamber for 180 s. Next day (training day), the mouse is placed in the chamber for 180 s and receives a mild foot shock (0.5 mA, 2 s) at 120 s and 150. On 1 day after foot shocks (Test day), a mouse is returned to the chamber for 180 s. Freezing time is recorded on habituation and retention days. **(B)** Freezing time is the percentage (%) of total time spent in the chamber. The number of mice used: WT ($n = 20$ that includes 10 male mice and 10 female mice) $Q2^{+/-}$ ($n = 20$ that includes 10 male mice and 10 female mice). *Post-hoc* Tukey test results are shown for the habituation day vs. the test day in WT ($^{\#}p < 0.001$) and in $Q2^{+/-}$ ($^{\wedge}p < 0.001$). **(C)** The average latency to tail-flick. In tail-flick assay, a mouse is restrained in a 50 ml conical tube and the tail is lower into a 36°C water bath and the latency to tail-flick is recorded for 30 s. The procedure is repeated with a 51°C water bath. All mice reached 30 s latency at 36°C. The number of mice used: WT ($n = 20$ includes 10 male mice and 10 female mice), $Q2^{+/-}$ ($n = 20$ includes 9 male mice and 11 female mice). Data represent the mean ± SEM. *Post-hoc* Tukey test results are shown for 36°C vs. 51°C in WT ($^{\#}p < 0.001$) and in $Q2^{+/-}$ ($^{\wedge}p < 0.001$). No significant difference between genotypes was found (n.s.). The individual data points are shown in **Supplementary Figure S2**.

an animal facility technician) might have contributed to this low performance of a small portion of male mice.

In addition, the female groups failed to show significant object location or novel object recognition as indicated by average DI not significantly different from zero (**Figure 3B**). Therefore, we concluded that female mice failed to display adequate performance on the task for analysis. Previous studies have shown that female mice trained outside the proestrus stage did not recognize the new object location 1 day after training whereas female mice trained in proestrus performed well in OLT similar to male mice (Gall et al., 2021). Although we did not monitor the estrous cycle in female mice, we speculate that their deficits in OLT and NORT might have resulted from different stages in their estrous cycle, which could have had varying effects on their exploratory behavior and/or spatial and object memory (Gall et al., 2021).

Because female mice failed to perform the OLT and NORT tasks, male mice were analyzed alone. Results of the *t*-test comparing DI for OLT and NORT in male mice showed no significant effect of genotype (**Supplementary Table S2**, **Figure 3B**). This result suggests that heterozygous loss of *KCNQ2*

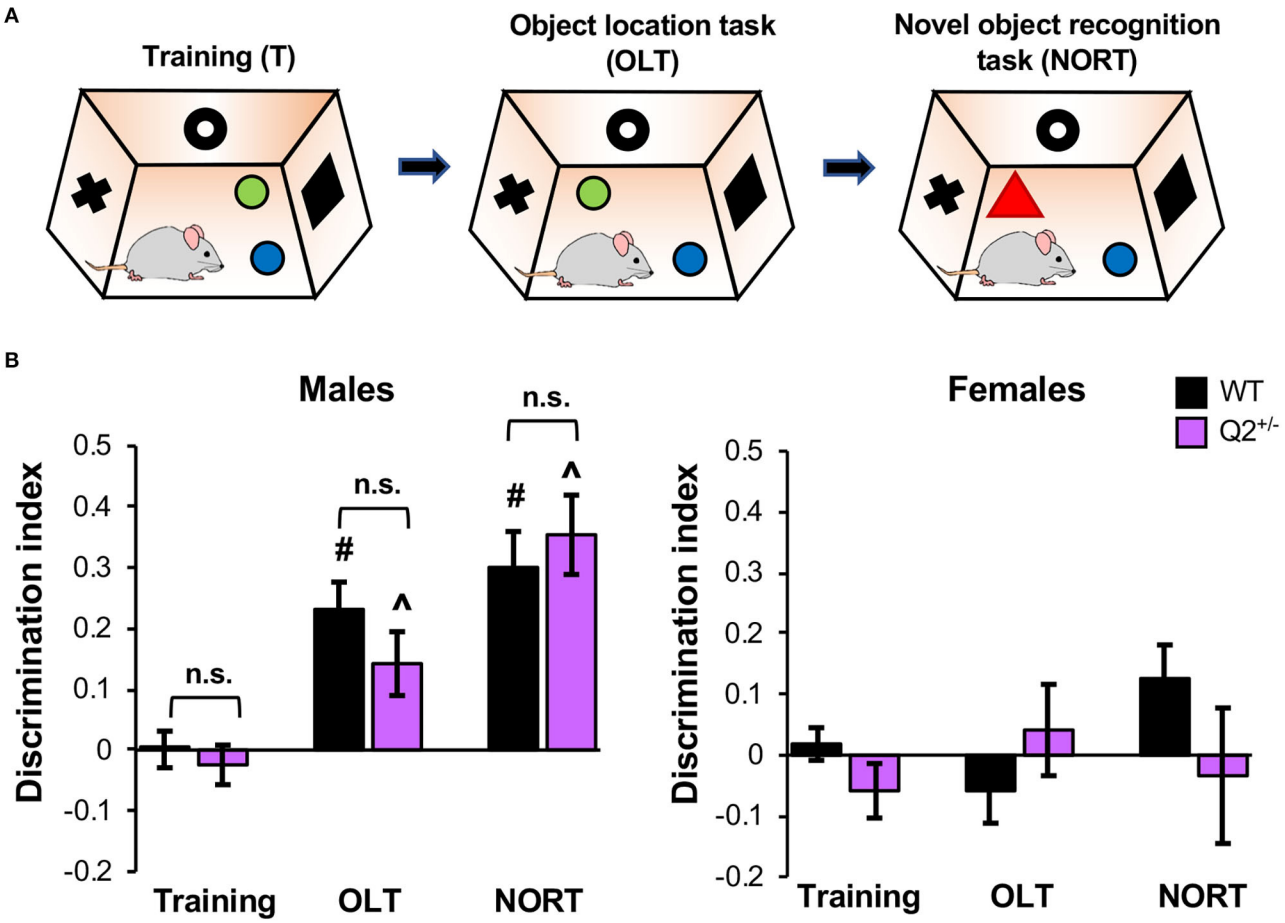

FIGURE 3 | Heterozygous loss of Potassium Voltage-Gated Channel Subfamily Q Member 2 (*KCNQ2*) does not affect object location and recognition memory in male mice. *KCNQ2*$^{+/+}$ (wild-type; WT) mice and *KCNQ2*$^{+/-}$ (Q2$^{+/-}$) mice at age 4–6 months were subjected to the object location task (OLT) and novel object recognition task (NORT). **(A)** The design of the OLT and NORT. **(B)** Discrimination index (DI) of male mice during OLT and NORT. During OLT and NORT, positive DI indicates the preference for the novel object or location, negative score equals preference for a familiar object or location, and zero score indicates no preference. The number of mice used: male mice (WT = 14, Q2$^{+/-}$ = 8), female mice (WT = 12, Q2$^{+/-}$ = 7). Data represent the mean ± SEM. *Post-hoc* Tukey test results are shown for training vs. OLT or training vs. NORT for WT male mice (#$p < 0.001$) and for Q2$^{+/-}$ male mice (^$p < 0.001$). The individual data points are shown in **Supplementary Figure S3**.

does not affect spatial memory and object recognition memory in male mice.

DISCUSSION

In this study, we performed behavioral phenotyping of the *KCNQ2*$^{+/-}$ mice, which lack half of the *KCNQ2* transcript and display reduced K_v7.2 expression but show no spontaneous seizures (Watanabe et al., 2000; Kim et al., 2019). Although *KCNQ2*$^{+/-}$ mice have been previously shown to display enhanced exploratory and repetitive behaviors and reduced sociability in both sexes as compared to *KCNQ2*$^{+/+}$ mice (Kim et al., 2019), this study provides the first evidence that heterozygous loss of *KCNQ2* does not affect fear-induced learning and memory on the IA task and CFC in both sexes (**Figures 1**, **2**) nor object location or recognition memories in male mice (**Figure 3**).

Interestingly, although both genotypes displayed a similar number of crosses to reach the criterion (**Figure 1D**), we observed that *KCNQ2*$^{+/-}$ mice took longer to reach the criterion during training especially in the fifth trial in the IA task than *KCNQ2*$^{+/+}$ mice (**Figures 1B,C**), suggesting that *KCNQ2*$^{+/-}$ mice tended to delay crossing to the dark chamber per trial compared to the WT mice. The interpretation of this difference is unclear. We speculate that *KCNQ2*$^{+/-}$ mice tend to delay crossing to the dark chamber per trial as compared to the WT mice, reflecting ambivalence in the decision-making during the phase of the fear-motivated learning (**Figure 1B**).

However, it is unclear how heterozygous loss of K_v7.2 could affect the decision-making during the phase of fear-motivated learning. Previous studies have shown that neonatal exposure to K_v7 opener retigabine and linopirdine does not affect basal nociceptive sensitivity of rats in the tail-flick assay (Frankel et al., 2016) and upon a foot shock (Cook et al., 1990), respectively. Application of K_v7 antagonist XE991 also does not increase

thermal hyperalgesia in a rat model of neuropathic injury (Rose et al., 2011). Similar to these pharmacological studies, we found that *KCNQ2*$^{+/-}$ mice displayed similar thermal nociception as the WT mice in tail-flick assay (**Figure 2C**). Thus, the effect of reduced I_M in this phase of decision-making could arise not from the difference in nociception. Rather, it could arise from hyperexcitability of the temporal lobe involved in passive IA because *KCNQ2*$^{+/-}$ mice show a heightened seizure susceptibility against kainic acid (Kim et al., 2019), which induces status epilepticus arising from the temporal lobe (Levesque and Avoli, 2013). Alternatively, considering that $K_v7.2$ and $K_v7.3$ are present in medial PFC (Pan et al., 2006), which suppresses amygdala outputs (Quirk et al., 2003), the slightly lengthened decision-making time in *KCNQ2*$^{+/-}$ mice could result from the increased activity of medial PFC by reduced $K_v7.2$ expression, which in turn inhibits the amygdala-dependent perception of fear (Etkin and Wager, 2007).

Minimal disruption of memory in *KCNQ2*$^{+/-}$ mice (**Figures 1–3**), which lacks spontaneous seizures (Watanabe et al., 2000; Kim et al., 2019), is in sharp contrast to cognitive deficits induced by genetic ablation of I_M in mice, which display spontaneous seizures (Peters et al., 2005; Milh et al., 2020; Kim et al., 2021). For example, dominant negative suppression of I_M by transgenic overexpression of the dominant negative mutant $K_v7.2^{G279S}$ or heterozygous expression of dominant negative EE mutants $K_v7.2^{T274M}$ and $K_v7.2^{M547V}$ in the developing brain induces spontaneous seizures, impaired hippocampus-dependent spatial memory, and object recognition memory (Peters et al., 2005; Milh et al., 2020; Kim et al., 2021). Considering that *KCNQ2* and *KCNQ3* expressions begin during embryonic development (Dirkx et al., 2020), we propose that K_v7 channels contribute to normal brain development and a significant reduction in K_v7 current early in the development may be necessary to disrupt proper circuit formation critical for cognition alone (Watanabe et al., 2000; Soh et al., 2014) or to induce spontaneous seizures, which could exacerbate this disruption.

In contrast to genetic models, pharmacological studies have revealed conflicting roles of K_v7 channels in learning and memory. K_v7 antagonist XE991 is shown to reduce the induction threshold of long-term potentiation (LTP) of excitatory synaptic strength at hippocampal CA1–CA3 synapses (Song et al., 2009; Fontan-Lozano et al., 2011), suggesting a facilitating role of K_v7 inhibition in LTP, which mediates hippocampus-dependent learning and memory (Whitlock et al., 2006). Consistently, K_v7 antagonists enhance fear memory and block memory impairments induced by hypoxia (Cook et al., 1990) and cholinergic depletion (Fontan-Lozano et al., 2011). In contrast, K_v7 channel opener retigabine is shown to inhibit the stress-induced reduction in hippocampus-dependent spatial memory (Li et al., 2014). These studies suggest that memory can be enhanced by either acute inhibition or enhancement of I_M depending on the underlying circuitry and the pathological condition. Given that the tauopathy mouse model of dementia displays reduced frontotemporal expression of K_v7 subunits (de Jong and Jepps, 2018), future studies shall further explore the precise role of I_M on age-related dementia and AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author. The data excel file is also available in figshare: https://doi.org/10.6084/m9.figshare.20073788.v1.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana Champaign.

AUTHOR CONTRIBUTIONS

HC and GT conceived of the study and participated in its design and coordination. HC, GT, and AW drafted the manuscript. GT and AW carried out the experiments and statistical analyses. JR contributed to the analyses and manuscript preparation. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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The effects of bilateral prostriata lesions on spatial learning and memory in the rat

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Area prostriata is the primary limbic structure for rapid response to the visual stimuli in the far peripheral visual field. Recent studies have revealed that the prostriata receives inputs not only from the visual and auditory cortices but also from many structures critical for spatial processing and navigation. To gain insight into the functions of the prostriata in spatial learning and memory the present study examines the effects of bilateral lesions of the prostriata on motor ability, exploratory interest and spatial learning and memory using the open field, elevated plus-maze and Morris water maze tests. Our results show that the spatial learning and memory abilities of the rats with bilateral prostriata lesions are significantly reduced compared to the control and sham groups. In addition, the lesion rats are found to be less interested in space exploration and more anxious while the exercise capacity of the rats is not affected based on the first two behavioral tests. These findings suggest that the prostriata plays important roles in spatial learning and memory and may be involved in anxiety as well.

KEYWORDS

prostriata, open field test, elevated plus-maze test, Morris water maze test, spatial learning and memory, anxiety, presubiculum

Introduction

Area prostriata (prostriata, Pro) was described in the brains of non-human primates (NHP) and humans over 50 years ago (Sanides, 1969; Allman and Kaas, 1971; Sousa et al., 1991; Barbas, 1993; Ding et al., 2003). Previous studies on the prostriata were mainly carried out in NHP including marmoset and macaque monkeys. However, the prostriata in the monkeys as well as in humans is located deep into the anterior calcarine fissure (Sanides, 1969; Morecraft et al., 2000; Ding et al., 2003, 2016) and this makes the prostriata difficult to be targeted with precise injections of neural tracers or lesion chemicals. Consequently, there are less data available about the brain-wide connections and functions of the prostriata. Its efferent projections were only found to reach to the

primary visual cortex (V1), association auditory cortex (A2), medial orbitofrontal cortex (ORBm), middle temporal area and cingulate motor area in the NHP brains (Sousa et al., 1991; Barbas, 1993; Rosa et al., 1993; Cavada et al., 2000; Morecraft et al., 2000; Falchier et al., 2010; Rockland, 2012) and no afferent projections were reported in NHP.

In 2013, the mouse equivalent of the prostriata was discovered (Ding, 2013). Recently, Ding et al. have also reported the homologous prostriata in the rats and brain-wide connections of the prostriata in both rats and mice (Chen et al., 2020, 2021; Hu et al., 2020; Lu et al., 2020). Briefly, the prostriata in the rats and mice receives its main inputs from the dorsal lateral geniculate nucleus (DLG), primary and secondary visual and auditory cortices and the cortical regions important for spatial processing and navigation such as subiculum (Sub), presubiculum (PrS; including dorsal PrS or postsubiculum; PrSd-PoS), retrosplenial cortex (RS), medial entorhinal cortex (MEC), anterior thalamic nuclei [ATN, including anterodorsal (AD), anteroventral (AV), anteromedial (AM), and laterodorsal (LD) thalamic nuclei] as well as from the contralateral prostriata (Ding, 2013; Chen et al., 2020, 2021; Ding et al., 2020; Hu et al., 2020; Lu et al., 2020). The efferent projections of the prostriata mainly reach to the V1, PrS-PoS and the subcortical regions that is important for visuomotor behaviors such as lateroposterior thalamic nucleus-pulvinar complex (LP-Pul), ventral lateral geniculate nucleus (VLG), pretectal nucleus (PTN), zone incerta (ZI), and pontine nucleus (PN) (Chen et al., 2021).

Previous studies of the NHP and human brains revealed that the prostriata plays an important role in the rapid processing and analysis of information from far peripheral visual field (Yu et al., 2012; Mikellidou et al., 2017; Tamietto and Leopold, 2018). This function is consistent with the strong and direct projections from the rostral DLG and medial V1 (both receiving inputs from peripheral visual fields) to the prostriata (Lu et al., 2020; Chen et al., 2021). However, our recent studies have also revealed moderate prostriata connections with auditory and olfactory cortices as well as strong connections with spatial memory system structures such as the Sub, PrS, RS, MEC, and ATN. These findings indicate that the prostriata may also play critical roles in multimodal sensory integration and spatial learning and memory. In addition, the prostriata strongly projects to the LP-Pul, which has strong projections to the amygdale, a critical structure for emotion and anxiety. Based on these findings, the first aim of the present study is to chemically damage bilateral prostriata in the rats and examine the effects of these lesions on spatial learning and memory ability. Our second aim is to study the effects of the prostriata lesions on the rat's anxiety behaviors. Our additional aim is to explore the effects of the lesions on neural activity in the downstream target regions of the prostriata partly because many previous studies showed that specific brain lesions lead to hypoactivity in some closely connected regions (Jenkins et al., 2006; Vann and Albasser, 2009; Dupire et al., 2013). In addition, neural activity in the downstream regions may also be affected by behavioral deficits in lesioned animals.

Abbreviations: A2, association auditory cortex; AD, anterodorsal thalamic nucleus; AM, anteromedial thalamic nucleus; ATN, anterior thalamic nucleus; AV, anteroventral thalamic nucleus; DLG, dorsal lateral geniculate nucleus; LD, laterodorsal thalamic nucleus; LP-Pul, lateroposterior thalamic nucleus-pulvinar complex; MEC, medial entorhinal cortex; NHP, non-human primates; ORBm, medial orbitofrontal cortex; PaS, parasubiculum; PN, pontine nucleus; PoS, postsubiculum; PrS, presubiculum; PTN, pretectal nucleus; RS, retrosplenial cortex; SN, substantia nigra; Sub, subiculum; V1, primary visual cortex; VLG, ventral lateral geniculate nucleus; ZI, zone incerta.

Materials and methods

Animals

Thirty-two adult Sprague-Dawley (SD) rats of both sexes (280–350 g, from the Beijing Vital River Laboratory Animal Technology Co., Ltd.) were used as experimental subjects. The number of male and female rats used in this study was not recorded. All the rats were kept in the same room with standard laboratory conditions (12 h light/dark cycle; setting temperature = 22 ± 2°C; setting humidity = 50 ± 10%), as well as free access to food and water in this present study. All experimental procedures were followed in accordance with the protocols that have been approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University.

Animal surgery

All animals were randomly divided into control group (no injection; n = 10), sham group (the prostriata was injected with 0.9% sterile saline; n = 10)and experimental group (the prostriata was injected with 10 mg/ml Ibotenic acid; n = 12). The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) before the operation. After the rats were completely unconscious, the rats were placed in a stereotaxic frame. After top-hair shaving and disinfection, a 2-cm midline incision was made on the top of the rats' head and the nose clip was adjusted to make the bregma and lambda at the same level. The specific location and layers of the prostriata in the rats have been recently identified (Chen et al., 2020; Lu et al., 2020). A suitable drill was used to make two holes (one per side) in skull over the target area and a 0.5 μl Hamilton syringe was used to deliver the injections (0.3 μl per side; each for 10 mins). The coordinates for all the prostriata injections are -8.72 (bregma), 3.20 (off midline), and 4.05 (depth). After the injections, the syringe was kept in place for another 10 mins and then slowly pulled out. After the wound suturing the rats were placed on a warm electric blanket until they woke before returning them to their cages. Two rats in the

sham group did not survive on the surgery day likely due to some issue with the anaesthetization.

Behavioral tests

After 10 days of postoperative recovery, the rats were brought to the behavioral testing room approximately 2 h before the tests. During the next 8 days, the rats were subjected to behavioral tests, including the open-field test (OFT; on the 11th day), elevated plus-maze (EPM; on the 12th day) test and Morris water maze (MWM; on the 13th–17th days) test. On the 18th day, the platform was removed from the pool and each rat was tested for 2 min to measure spatial learning and memory capacity. All the rats were sacrificed using the same procedure (see Brain tissue preparation below) immediately after the last behavioral test.

Open-field test

The apparatus with a volume of 100 cm × 100 cm × 40 cm (length × width × height) was evenly divided into 25 squares (20 cm × 20 cm). The 25 identical squares were marked as 1–25 from right to left starting from the bottom right corner, of which squares 7, 8, 9, 12, 13, 14, 17, 18, and 19 were set as the area of center (Hu et al., 2017). In a dark, quiet and well-ventilated room, the rats were placed in order from area 1 head down and allowed to explore for 5 mins. After each test, the apparatus was cleaned and disinfected with 75% alcohol before testing the next rat. The whole experiments were recorded by video, and the related software was used for data analysis at the end of the experiments.

Elevated plus-maze test

The apparatus consists of two open arms (length × width = 50 cm × 10 cm), two relatively closed arms (length × width × height = 50 cm × 10 cm × 40 cm) and a central platform (length × width = 10 cm × 10 cm) connected to four arms. The open arms, closed arms and area of center were all black and were perpendicular to each other (Bruijnzeel et al., 2019; Knight et al., 2021). The plus maze was fixed on a cross bracket with the same length as its arms, and the cross bracket was 50 cm above the floor. In a dark, quiet and well-ventilated room, the rats were placed in order from the central area head down and allowed to explore for 5 mins. The staying time of the rats in the open arms, the closed arms and the central area as well as the number of times the rats entered the open arms were recorded. After each test, the feces were removed after each trial and disinfected with 75% alcohol.

Morris water maze test

The Morris Water Maze (MWM) (120 cm in diameter, 50 cm in height) was placed in a quiet and well-lit room (Warner et al., 2013). The inner wall of the MWM was painted black and has four typical patterns on it, including triangles, crosses, circles, and squares, to help the rats remember the position of the platform. The circular pool was divided into four quadrants and a circular platform with a diameter of 11 cm was placed in the center of the first quadrant (between the triangle and square), and then 25 cm-deep water was poured into it (the height of the water surface just immersed the platform 2–3 cm; water temperature = 22 ± 2°C). A high-definition camera was placed on the top of the MWM to record the trajectories of the rats, which were analyzed with the EthoVision XT 14 system. Since the rats tended to stay in a dry environment and hated staying in water, they would rush to find the platform to stay away from the water. For the next 6 days, the rats were trained to find the platform, but on the sixth day the platform was removed from the pool to test animals' memory of the platform (Sun et al., 2021). In the first 5 days, the rats were tested four times a day, each time they were put into the pool from the center of a different quadrant and each rat was gently released along the maze wall into the pool in the four quadrants every day (**Table 1**). The rats were allowed to train for a maximum of 2 mins each time (Vorhees and Williams, 2006). If the rat found the platform within 2 mins, it was allowed to stay on platform for 3 s and then take it out. If the rat could not find the platform in 2 mins, the rat would be guided to find the platform and allowed to stay on the platform for 10 s. After each rat completed the training, the rat was wiped dry and placed in a cage with suitable temperature to rest for 20 mins (Zhang et al., 2015). On the sixth day, the platform in the first quadrant was removed from the pool, and each rat was placed in the pool from the center of the third quadrant and explored for 2 mins. We chose the third quadrant, the farthest from the platform, as the quadrant to release the rats into the pool, to reduce the possibility for the rats to find the platform location by chance.

TABLE 1 Summary of the starting position of each training (hidden platform located at NE).

Day	Trial 1	Trial 2	Trial 3	Trial 4
1	SW	SE	NW	NE
2	SE	NE	SW	NW
3	NW	SW	NE	SE
4	NE	NW	SE	SW
5	SW	NW	SE	NE
6 (Probe)	SW			

Brain tissue preparation

After completing a series of behavioral experiments, the rats were deeply anesthetized with an overdose of sodium pentobarbital (60 mg/kg, i.p) until they were completely unconscious and then perfused transcardially with 0.1 M phosphate buffer (PB, PH = 7.3) followed by 4%

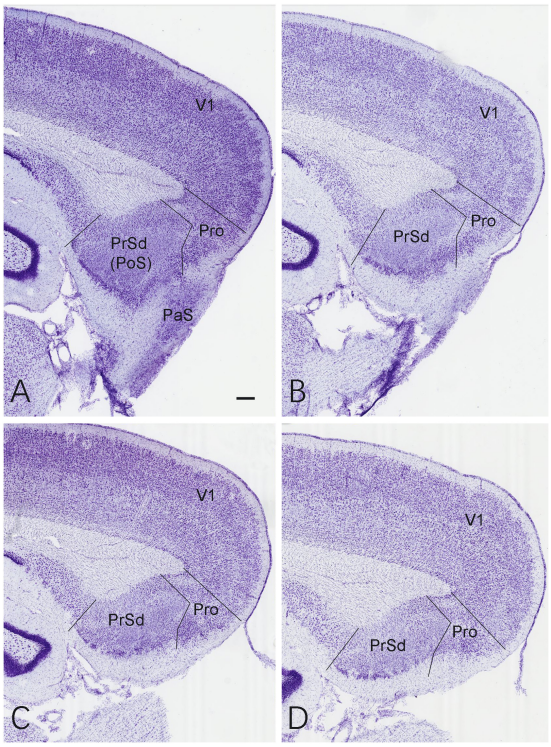

FIGURE 1
Location and normal cytoarchitecture of the rat prostriata. **(A–D)** Sequential Nissl-stained sagittal sections from the lateral **(A)** to medial **(D)** levels of the prostriata and adjoining regions in a normal (control) rat. Bar in panel **(A)**: 200 μm for all panels.

paraformaldehyde (PFA) in chilled PB. After the rat's liver turned white and hardened, the brain was taken out and postfixed in 4% PFA at 4°C overnight. For the next 3–4 days, the brain was stored in 15 and 30% sucrose, in sequence, until the brain sank to the bottom of the bottle. The brain was cut into the left and right hemispheres along the midline, and then the hemispheres were sectioned into 40 μm thick sequential sagittal sections with a freezing microtome (Leica CM3050 S). The brain sections were stored in cryoprotectant for subsequent experiments (Chen et al., 2020; Lu et al., 2020).

Nissl stain

The sections containing the prostriata were selected from the cryopreservation solution and rinsed with PB. The sections were then mounted on slides and dried in an oven at 37°C. For Nissl staining, the sections were placed in xylene and gradient alcohol (100, 95, 85, and 70%) for 5 mins each and then stained in 0.1% Cresyl Violet solution for 20 mins and immersed in distilled water for 5 mins. Finally, the sections were dehydrated in 85, 95, and 100% ethanol for 5 mins each before being placed in xylene (two times) and coverslipped.

Immunohistochemistry

The sagittal sections containing the major downstream target regions of the rat prostriata were selected for c-fos IHC. These regions include the PrSd-PoS, LD, LP-Pul, PTN, and VLG and are directly innervated by the prostriata (Chen et al., 2021). In addition, we also evaluated c-fos expression in zona incerta (ZI) and substantia nigra (SN), which are not the targets of the prostriata. Selected sections were rinsed three times with 0.1 M PB and immersed in 0.3% hydrogen peroxide for 10 mins. Then the sections were rinsed three times again and blocked in 5% BSA for 60 mins. At the end, the sections were incubated with primary antibody against c-fos (200 μg/ml, 1:200, Boster Biological Technology, Wuhan, China) diluted with the 0.1 M PB overnight at 4°C. On the next day, the sections were incubated in the secondary antibodies (biotinylated goat anti-mouse/rabbit IgG, Boster) for 60 mins after thorough rinse with 0.1 M PB. The sections were then rinsed again and immersed in the Streptavidin-Biotin Complex solution (SABC kit, Boster Biological Technology) for 60 mins. After rinse with 0.1 M PB, the sections were incubated in 3, 3-diaminobenzidine (DAB) solution for 3 mins in a dark environment. Finally, the sections were mounted on the slides, dehydrated and coverslipped.

Image capture and data analysis

All behavioral tests, including OFT, EPM, and MWM, were recorded and collected by the EthoVision XT 14 system. The movement trajectories of the rats were recorded by high-definition cameras. Statistical analyses were performed using the IBM SPSS 20.0 software. The performance of the animals during OFT, EPM, and MWM was analyzed using ANOVAs. The results in the graphs were presented by mean values ± standard deviations (mean ± SD). In all statistical analyses, $p < 0.05$ was considered significant. The Nissl- and IHC-stained sections were digitized with a scanner (Aperio CS2, Leica). Cell counts of c-fos positive neurons (for neuronal activity) in selected brain regions (i.e., PrSd-PoS, LD, LP-Pul, PTN, VLG, ZI, and SN) was performed using image J software and the statistics was done using one-way ANOVA.

Results

Localization of the lesions in the prostriata

The location and extent of the prostriata in rats have been demonstrated recently in both Nissl and calbindin-D28k (CB) stained sections (Lu et al., 2020; Chen et al., 2021). As reported (Lu et al., 2020), the prostriata is a limbic cortex lacking granular

layer 4 and having relatively larger cells in its superficial layers 2–3 compared to adjoining regions and is located at the junction among the PrSd-PoS, RS, parasubiculum (PaS), and the medial visual cortex (see Figure 1).

Based on the location of the prostriata (Lu et al., 2020; Chen et al., 2021), the extent of the chemical lesions caused by ibotenic acid was evaluated on Nissl-stained sagittal sections of both hemispheres from sham and experimental groups. Compared to the control group (Figure 1), no significant cell loss was observed in the prostriata of the sham group (e.g., Figures 2A,B) although a small number of cells were lost along the needle track (indicated by the arrow in Figure 2B). However, in the experimental group, all animals included for data analysis display bilateral lesions in the prostriata and the lesions were severe and many cells in the prostriata were lost, as shown in the sequential sagittal sections from the lateral to medial levels (the damaged prostriata areas appear pale; see the Pro in Figures 2C–F). In addition, a small portion of the PaS and/or layer 6 of the overlying V1 (indicated by the # in Figures 2C,D) also showed some cell loss, but most of the PrSd-PoS and PaS regions were found to be intact (Figures 2C,D). It should be mentioned that two rats (from experimental group) were excluded from data analysis because the injections missed the target and spared over 70% of the prostriata. In other 10 rats of this group, the injections consistently damaged 70–90% of the prostriata of each hemisphere (e.g., Figures 2C–F).

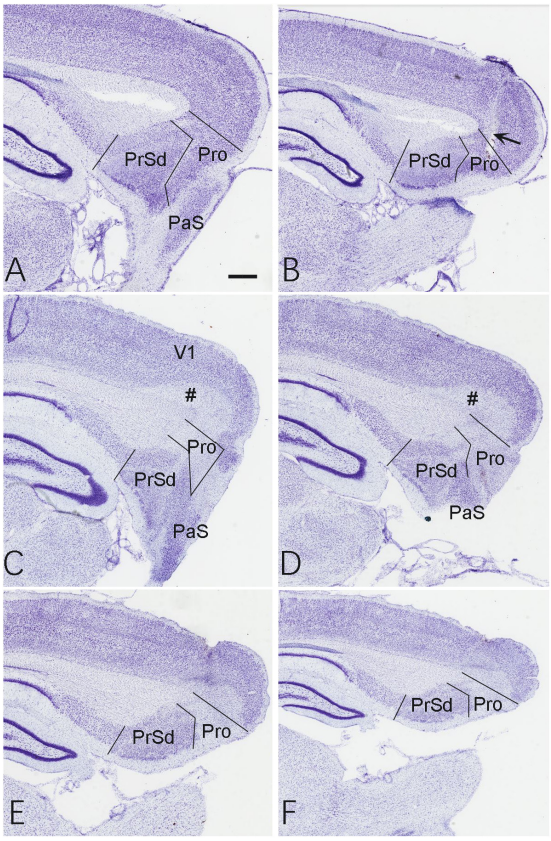

FIGURE 2
Evaluation of the lesion and its extent. **(A,B)** Two Nissl-stained sagittal sections from the lateral **(A)** and middle **(B)** levels of the prostriata and adjoining regions in the sham group. The arrow in panel **(B)** indicates a needle track of the injection. **(C–F)** Sequential Nissl-stained sagittal sections from lateral **(C)** to medial **(F)** levels of the prostriata and adjoining regions in the experiment group. The #s in panels **(C,D)** indicate layer 6 of the V1. Bar in panel **(A)**: 400 μm for all panels.

Behavioral tests

Open-field test

We used the classic open-field test (OFT) (Figure 3A) to assess the locomotor ability of the rats and their interest in exploring space (Hu et al., 2017), and one-way ANOVA for the statistics, which found a significant effect of the group (control, sham and experiment) on time spent in the central area ($F[2, 24] = 22.47$, $p < 0.0001$, [eta-squared: 0.6518]). Post-hoc comparison (Tukey HSD, $a = 0.05$) revealed that the time spent in the central area was significantly reduced in the experimental group compared to both the control and sham groups. Comparison of the control ($N = 10$), sham ($N = 8$) and experiment ($N = 10$) groups showed no significant change in the movement speed ($F[2, 24] = 3.136$, $p = 0.0617$, [eta-squared: 0.2072]). Post-hoc comparison (Tukey HSD, $a = 0.05$) revealed that there is also no significant difference in the movement speed between each two groups (see Figure 3B). Comparison of the movement trajectories (paths) of the control, sham and experiment groups showed that the experiment group were less interested in exploring the center area than the other groups (Figure 3C). Compared with the control group and the sham group, the times spent in the central area was significantly reduced in the experimental group (Figure 3D).

Elevated plus-maze test

The elevated plus-maze (EPM) (Figure 4A) was used to assess anxiety of the rats in the control ($N = 10$), sham ($N = 8$), and experiment ($N = 10$) groups, and one-way ANOVA was used for statistics. It was observed that most of the movement trajectories of the rats were in the closed arms (Figure 4B), but the rats in the control and sham groups explored more in the open arms. The times spent in the open arms for the experimental group was significantly reduced compared to the control and sham groups ($F[2, 25] = 5.30$, $p < 0.0121$, [eta-squared: 0.2977]) (Figure 4C). Post-hoc comparison (Tukey HSD, $a = 0.05$) showed no significant changes between the sham and control groups (Figure 4C). As for the frequency of the rats entering the open arms, experimental group entered the open arms less frequently than those in the control or sham groups ($F[2, 25] = 5.71$, $p = 0.0091$, [eta-squared: 0.3136]) (Figure 4D). Post-hoc comparison (Tukey HSD, $a = 0.05$)

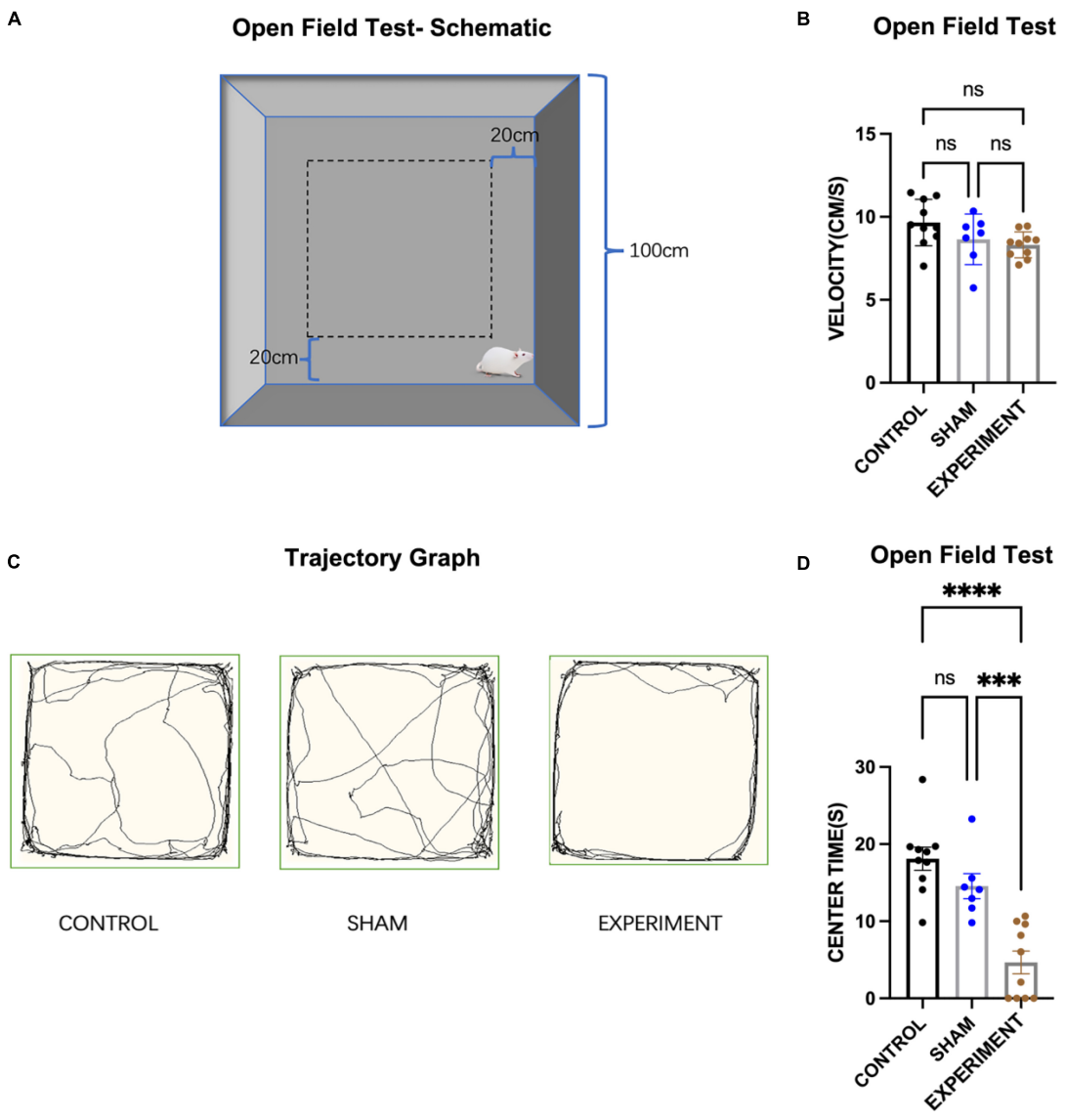

FIGURE 3
Open-field test. **(A)** Schematic diagram of the open field test. **(B)** The movement speeds of the rats (mean ± SD). **(C)** Movement trajectory paths of the rats. **(D)** The times spent in the central area (mean ± SD). ***$p < 0.001$, ****$p < 0.0001$.

revealed no significant changes between the sham and control groups (Figure 4D).

Morris water maze test

The Morris water maze (MWM) was used to evaluate spatial learning and memory of the rats (Figure 5A), and one-way ANOVA used for statistics. From the movement trajectory, it was observed that the rats found the platform more quickly after training in the first 5 days (Figure 5C). The average time to reach the platform in the first 5 days in the control ($N = 10$), sham ($N = 8$), and experimental ($N = 10$) groups all decreased with the increase of training time (Figure 5B). After removing the platform on the sixth day the experimental group stayed on the original platform region for less time than the control and sham groups (Figure 5D) ($F[2, 25] = 11.01$, $p = 0.0004$, [eta-squared: 0.4684]). In addition, since the platform was in zone 1, we also calculated the times the rats spent in this zone. The experimental group stayed in zone 1 for less time than the control and sham groups (Figure 5E) ($F[2, 25] = 6.653$, $p = 0.0048$, [eta-squared: 0.3474]). Improvements in escape latency across the 5 days of MWM training were analyzed with a mixed-model ANOVA with Group as a between-subjects factor and Day (1–3) as a between-subjects factor. The statistics shows that Day $F(3.060, 76.51) = 17.41$, $p < 0.0001$; Group $F(2, 25) = 0.3075$, $p = 0.7380$; interaction (group × day) $F(8, 100) = 1.114$, $p = 0.3601$. Although the interaction is not

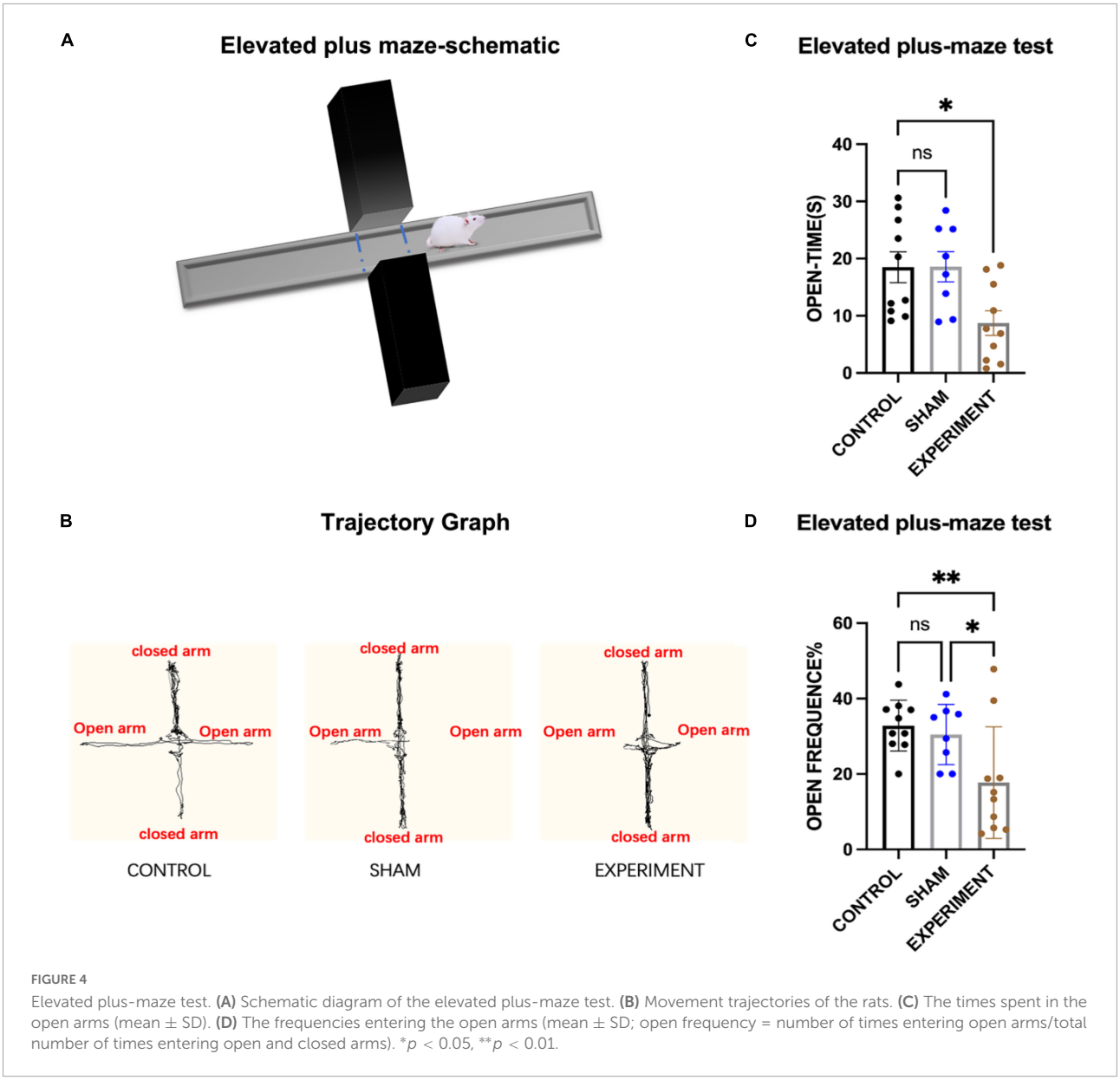

FIGURE 4
Elevated plus-maze test. **(A)** Schematic diagram of the elevated plus-maze test. **(B)** Movement trajectories of the rats. **(C)** The times spent in the open arms (mean ± SD). **(D)** The frequencies entering the open arms (mean ± SD; open frequency = number of times entering open arms/total number of times entering open and closed arms). *$p < 0.05$, **$p < 0.01$.

significant and this may suggest that the trend over days did not differ significantly by group, our results from the sixth day (the platform was removed) show that experiment group stayed on the original platform region for less time compared to other two groups (**Figure 5D**), suggesting at least the trend of the deficits in learning and memory over days. Finally, using the first 2 days as short-term memory practice (Bruszt et al., 2021), we found that the short-term spatial memory in the experimental group was not significantly affected (**Figure 5B**).

C-fos expression in downstream targets of the prostriata

Our previous studies revealed that the major target regions of the prostriata in rats include the PrSd-PoS, LD, LP-Pul, PTN, and VLG (Chen et al., 2021). Many previous investigators used c-fos expression as a tool to indicate activity of activated neurons (Sagar et al., 1988; Dragunow et al., 1989; Herrera and Robertson, 1996; Velazquez et al., 2015). In addition, c-fos expression was also used to indicate hypoactivity in closely connected regions after specific brain lesions and behavioral tests including learning and memory tests (Herrera and Robertson, 1996; Jenkins et al., 2006; Vann and Albasser, 2009; Dupire et al., 2013). Since the rats were sacrificed immediately after the probe trial in the MWM, neural activity in the target regions of the prostriata could be affected by both the behavioral deficits and loss of afferents, which could cause the hypoactivity. Therefore, we examined c-fos expression in the major target regions to evaluate the effects of the prostriata lesions on the

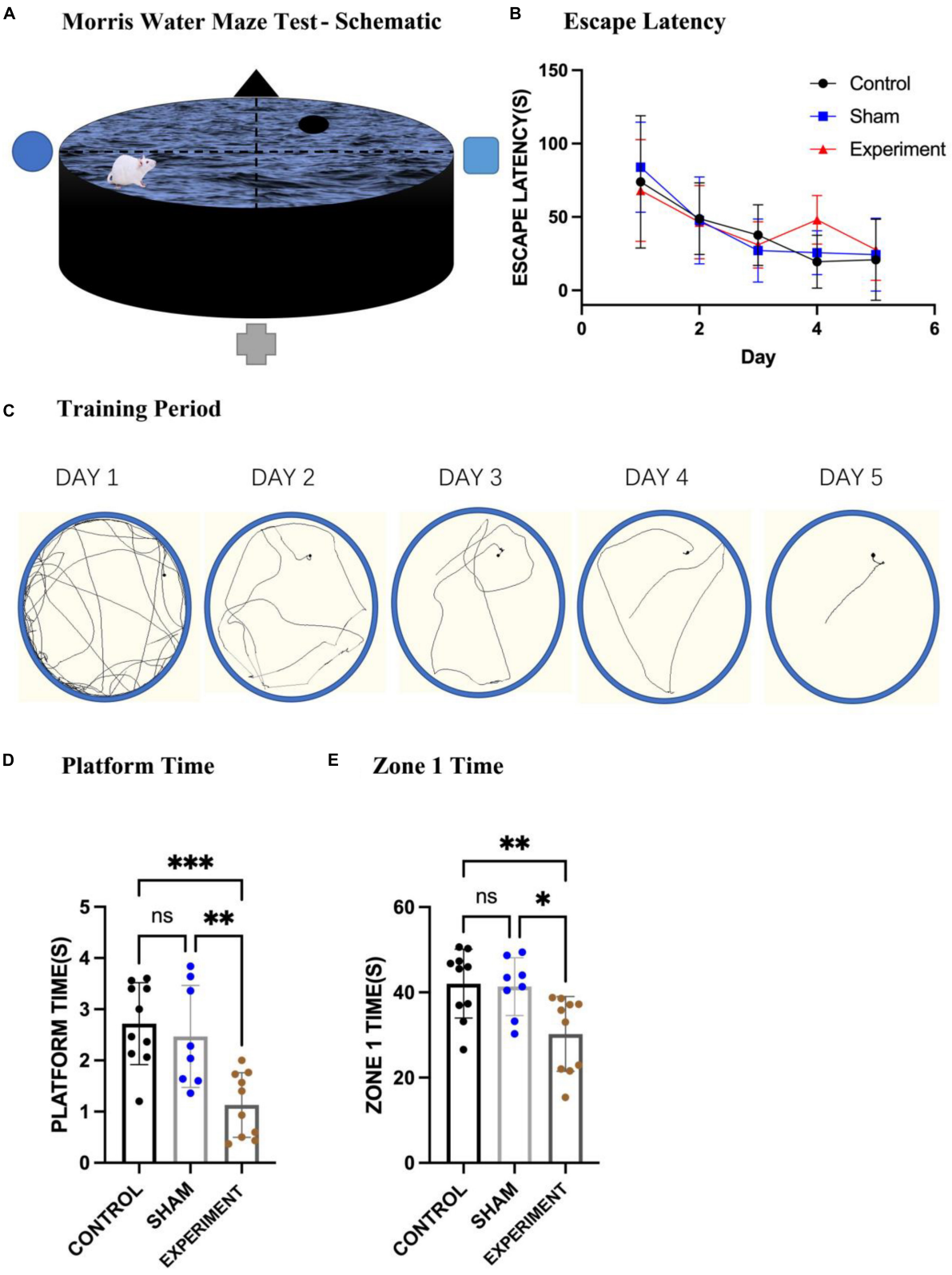

FIGURE 5
Morris water maze test. **(A)** A schematic of the MWM apparatus. **(B)** The mean times of the three groups swimming to the platform (mean ± SD). **(C)** Changes in locomotion trajectories of the rats reaching to the platform during the 5-day training period. **(D)** The times spent in the original platform area after the platform was removed (mean ± SD). **(E)** The times stayed in zone 1 on the sixth day of the test (mean ± SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

target regions. Targeted sections from three rats in each of the sham and experiment groups were randomly selected for c-fos IHC staining, and student *t* test was used for analysis. The results showed that the ratios of the number of c-fos positive neurons in the PrSd-PoS ($P < 0.01$), LD ($P < 0.05$), and LP-Pul ($P < 0.05$) was significantly reduced in experimental group compared to sham group (**Figures 6A–C**). However, the ratios of the number of c-fos positive neurons in the PTN ($P > 0.05$) and VLG ($P > 0.05$) of the experimental group did not show significant decrease although the ratios tended to decrease (**Figures 6D,E**). Finally, we also evaluated c-fos positive neurons in the ZI and SN that are not the targets of the prostriata. The ratios of the number of c-fos positive neurons in the ZI and SN of the experimental and sham groups did not show significant changes (e.g., **Figure 6F** for SN).

Discussion

The present study has showed that, following the OFT, EPM, and MWM tests, control and sham rats were able to maintain normal anxiety state and use spatial land markers to memorize specific locations. However, the experimental group with bilateral prostriata lesions displayed deficits in spatial learning and memory as well as possible anxiety, compared to control or sham groups. There was not significant effect on motor abilities of the lesion rats. However, we cannot completely rule out the possibility that some vision impairments may occur in some rats with some lesion in layer 6 of the V1 (see "Results" section). V1 is a large region and its layer 6 is mainly the region that initiates feedback projections to the DLG and thus would not significantly affect vision perception. Therefore, we believe that vision impairments, if any, are minimum or not significant in the present study. This conclusion is supported by our findings that the lesion rats did not show significant decrease in moving velocity in the OPT compared to control and sham groups and that the three groups did not show significant difference in escape latency during the first three days of the MWM training. These findings cannot be explained if the lesion rats had significant vision impairments. Taken together, we believe the behavioral changes observed in this study is the results of increased anxiety and deficits in spatial learning and memory.

Prostriata and spatial learning and memory

In this study, we used the neurotoxin ibotenic acid to damage neurons in the prostriata. The lesion caused by single injection covers most of the prostriata (**Figures 2C–F**) since the prostriata in rodents is small in size (Lu et al., 2020; Chen et al., 2022). Our results showed that the movement speeds of the rats in the control, sham and experimental groups did not show significant changes, so there was no significant difference in the movement ability among the three groups. We conducted escape training for each group for 5 days and found that the rats in all groups succeeded in finding the platform, suggesting the acquisition of spatial learning by the end of the training period even in the experiment group. However, when we took the first 2 days as short-term memory practice and the last 2 days as long-term memory one (Bruszt et al., 2021), we found that the short-term spatial memory in the experimental group was not significantly affected while the long-term spatial memory ability changed (**Figure 5B**). These results indicate that it is difficult for the rats in the experimental group to find the platform area on later days even they can find the platform area on the first day. Therefore, it is possible that bilateral damages to the prostriata reduce not only the spatial navigation ability but also their memory ability. We also counted the times the rats stayed in zone 1, where the platform was located and found that the times also decreased for the rats in the experimental group (**Figure 5E**). This may indicate that the prostriata-lesion rats had poorer ability to recognize precise and imprecise positions. Because the prostriata plays an important role in analysis of information from peripheral visual field (Rockland, 2012; Yu et al., 2012; Mikellidou et al., 2017; Tamietto and Leopold, 2018), the rats in the experimental group are likely not good at using the signs on the walls of the MWM apparatus compared to the other two groups.

Possible neural mechanisms underlying the spatial learning and memory impairment

Our recent studies have revealed that the prostriata receives direct projections from the visual cortex, AD, AV, LD, RS, Sub, PrSd-PoS, and MEC (Ding, 2013; Ding et al., 2020; Hu et al., 2020; Lu et al., 2020; Chen et al., 2021). All these structures are important components of spatial memory processing system and the AD and PrSd-PoS contain many head direction cells (Cho and Sharp, 2001; Hafting et al., 2005; Taube, 2007; Tsanov et al., 2011). It is likely that damage to the prostriata would impair the processing of spatial information participated by these structures such as landmark signal integration and accurate visual navigation.

On the other hand, the prostriata has strong projections to the PrS-PoS (Chen et al., 2021), which heavily innervates the MEC. The MEC contains many head direction cells, place cells and grid cells with the latter two function as grasping the location and integrating spatial information, respectively (Taube, 2007; Moser et al., 2008). Place cells are usually believed to help animals reach the target position by comparing the similarity between the current position and

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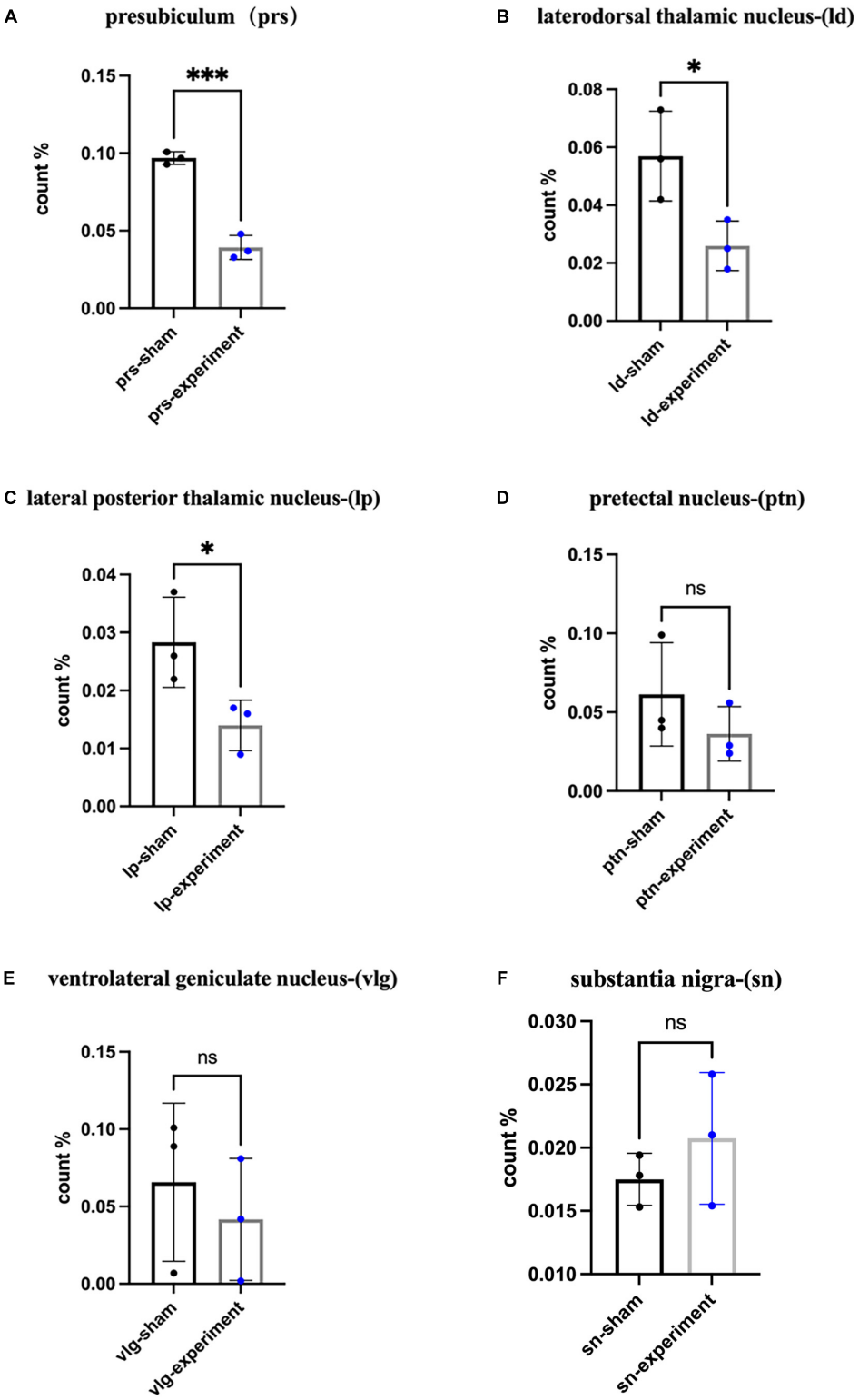

FIGURE 6
The percentage of c-fos expressing cells in the target areas. **(A)** The ratios of the number of c-fos expressing cells in the PrS-PoS (mean ± SD, $t_4 = 11.43$, $p = 0.0003$). **(B)** The ratios of the number of c-fos positive cells in the LD (mean ± SD, $t_4 = 3.03$, $p = 0.0388$). **(C)** The ratios of the number of c-fos expressing cells in the LP-Pul (mean ± SD, $t_4 = 2.78$, $p = 0.0494$). **(D)** The ratios of the number of c-fos expressing cells in the PTN (mean ± SD, $t_4 = 1.17$, $p = 0.3065$). **(E)** The ratios of the number of c-fos expressing cells in the VLG (mean ± SD, $t_4 = 0.64$, $p = 0.5551$). **(F)** The ratios of the number of c-fos expressing cells in the SN (mean ± SD, $t_4 = 1.00$, $p = 0.3739$) (ratio = the number of c-fos positive neurons/size of area examined for each structure). $^*p < 0.05$, $^{***}p < 0.001$.

the target position (Burgess and O'Keefe, 1996; Bush et al., 2015). Grid cells are thought to integrate both location and direction information and to provide a path integration input to place cells (Hafting et al., 2005; O'Keefe and Burgess, 2005; McNaughton et al., 2006; Rolls et al., 2006; Solstad et al., 2006; Taube, 2007). Therefore, it is possible that the prostriata may function as a node integrating visual landmark information, head direction and position information. When the prostriata is damaged, the functions of head direction cells, place cells and grid cells in the downstream target regions such as the PrSd-PoS, LD, and MEC would be impaired. This appears reflected in the reduction of c-fos positive neurons in some of the downstream structures such as the PrSd-PoS and LD.

Prostriata and anxiety

According to the results of the OFT, the times spent in the central area for the lesion group was significantly reduced in comparison with the other groups. According to this result, we could infer that the rats in the experiment group had decreased interest and even feared in the exploration of a novel environment (Hu et al., 2017). Our recent studies in rodents have found that the prostriata connects to the LP-Pul and ORBm (Hu et al., 2020; Chen et al., 2021). The LP-Pul, which receives strong and direct projections from the prostriata, were reported to be important in fear processing and in activating stress responses via its connections with amygdala (Goosens and Maren, 2001; Herman et al., 2005; Arend et al., 2008; McFadyen, 2019). The ORBm also plays an important role in emotional changes, including reward, aggression, and aversion (Butter et al., 1970; Butter and Snyder, 1972; Rolls, 2019). Therefore, we speculate that the rats with prostriata lesion may be less interested in exploring the environment and display increased anxiety due to the impairment of the prostriata and reduction of the afferents to the LP-Pul and ORBm. In addition, the prostriata belongs to and connects heavily with the limbic system, whose impairment could also increase anxiety and fear (Meyer et al., 2012; Deal et al., 2016). The possible fear and anxiety of the experimental group were also reflected in the EPM test. In this study we found that the times and frequencies of the lesion rats entering the open arms were less than those in the other two groups. Due to anxiety and/or lack of interest, the rats in the experimental group could be reluctant to explore relatively open area in both the OPT and EPM tests. In addition to possible emotional changes affecting the behavioral performance of the rats, we cannot ignore the impact of the reduced ability of the lesion rats to analyze the information from the peripheral visual field. Since the prostriata plays an important role in analyzing information from the peripheral visual field, the ability of the rats with damaged prostriata to explore in a distant unfamiliar environment would also be reduced (Yu et al., 2012; Mikellidou et al., 2017; Tamietto and Leopold, 2018; Lu et al., 2020; Chen et al., 2021). Therefore, we speculate that the changes in the behavioral performance of the rats following bilateral prostriata lesions could be the result of the simultaneous effects on the emotional and visual abilities of the rats.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by Institutional Animal Care and Use Committee of Guangzhou Medical University.

Author contributions

S-LD: conceptualization. S-YZ, J-YZ, C-HC, X-JX, and H-RC: data generation. S-YZ and S-LD: data analysis and manuscript writing. S-QC and SL-D: supervision. All authors have read and approved the submitted manuscript.

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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.

Publisher's note

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Preliminary investigation and application of a modified objects memory test in perioperative cognitive evaluation

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Objective: To investigate the applicability of a modified verbal learning test redesigned from the memory subtest of the Syndrom Kurztest (SKT) in perioperative cognitive evaluation.

Methods: Patients receiving elective herniorrhaphy and their accompanying family members (set as normal controls), 55–75 years old, were randomly divided into two groups. The two groups received the self-made objects memory test derived from the SKT (SMOT) SMOT or a traditional auditory verbal learning test (AVLT). The cognitive evaluation was administered at the bedside on the day before surgery and the second day after surgery.

Results: The SMOT test was administered to 121 subjects, while 107 patients received the AVLT test. After confirming that there was no significant difference in cognitive function between patients and their family members, the results of the SMOT and AVLT tests were compared. The results showed that the "low-score" ratio of the SMOT was significantly lower than that of the AVLT test ($P < 0.05$), and the influencing factors of the SMOT were less than those of the AVLT test. However, the learning effect of the SMOT was more significant ($P < 0.05$).

Conclusion: This study preliminarily confirms that the SMOT has better applicability to elderly Chinese individuals than AVLT in perioperative cognitive evaluation, but its learning effect should be noted.

KEYWORDS

verbal learning test, Syndrom Kurztest, postoperative cognitive dysfunction, elderly, neuropsychological test

1. Introduction

Postoperative cognitive dysfunction (POCD) occurs frequently in elderly patients undergoing major surgery. The assessment and diagnosis of POCD require the use of a combination of neuropsychological tests (Hanning, 2005; Evered and Silbert, 2018). The Auditory Verbal Learning Test (AVLT) is one of the classical neuropsychological tests used to assess learning and memory and is widely used for cognitive function assessment (Moller et al., 1998; Rasmussen et al., 2005). Different versions of AVLT have also been widely used in POCD studies, including the International Study Group of Postoperative Cognitive Dysfunction (ISPOCD) (Guo et al., 2007; Zhao et al., 2015).

The Chinese version of the AVLT has multiple versions, such as the Shanghai Mental Health Center version and the Chinese University of Hong Kong version, among which the Huashan Hospital version is widely used in cognitive assessment-related studies in China and has been confirmed to have good reliability and validity (Guo et al., 2007; Zhao et al., 2015). However, our research team found in the study that AVLT is difficult for elderly patients with complications, such as a low level of education, various dialects, and hearing impairment, which predispose subjects to give up halfway through the test. Therefore, we intend to develop a memory assessment scale with better applicability. The brief cognitive ability test (Erzigkeit's short cognitive performance test), also known as the Syndrom Kurztest (SKT), a cognitive assessment composite scale developed by the German researcher Erzigkeit H, is widely promoted internationally (Choi et al., 2004; Flaks et al., 2009) and is also recommended for POCD assessment (Rundshagen, 2014). The SKT consists of 9 subtests, of which subtests 1, 2, 8, and 9 are part of the memory test (SMOT), which uses cartoon-style pictures containing 12 objects, and the SMOT is less affected by factors such as cultural differences and level of education than AVLT (Choi et al., 2004; Flaks et al., 2009; Rundshagen, 2014). Based on the above background, our research team developed a modified version of the SMOT according to the SMOT picture memory evaluation method and AVLT scoring method, and this study mainly explored its applicability in the elderly population with a low educational level in China (Lu et al., 2021).

2. Subjects and methods

2.1. Subjects

This study was approved by the Medical Ethics Committee of our hospital and was a cross-sectional observational study. Inclusion criteria were as follows: patients ages 55 to 75 undergoing elective herniorrhaphy in our hospital from 1 March 2019 to 31 October 2021, their accompanying family members, and a willingness to sign informed consent. Exclusion criteria were as follows: inability to communicate effectively in Mandarin; inability to undergo spinal anesthesia due to objective or subjective factors; American Society of Anesthesiologists (ASA) $\geq$ III; history of central nervous system disease or mental illness; history of malignant tumors; severe chronic diseases (severe heart disease, lung disease, chronic neuralgia and other diseases, or disabilities affecting the quality of life); Mini-Mental State Examination (MMSE) score $<$ 20 points; patients who did not receive spinal anesthesia on the day of surgery; operation times of more than 2 h; serious complications (intraoperative hemodynamic instability, postoperative vomiting, headache, insomnia and delirium); any subjective or objective factors that interrupted the test.

The included subjects were randomly divided into the SMOT and AVLT groups. The SMOT group received the SMOT test twice on the day before surgery and the second day after the operation, while the AVLT group received the AVLT test at the same time point. Randomization method: An on-site lottery was performed in the consultation room before anesthesia, and randomization was performed by using the Excel function "Randbetween (1, 2)." If the value was "1," the patient was included in the SMOT group, and the accompanying family member was included in the AVLT group; if the value was "2," the reverse was true. If there were no accompanying family members, only the patient was randomized. The evaluation site was the consultation room before anesthesia, and the time limit for surgery was 15:00–18:00. For the convenience of the study, the evaluators included two young male doctors who could skillfully use the MMSE scale, modified SMOT and AVLT. Evaluator A assesses the patient, while evaluator B assesses the patient's family member. To ensure the consistency and proficiency of the two assessors in the operation of the guided language of the cognitive assessment measurement form, the two assessors successively pretested the MMSE scale and the SMOT and AVLT immediate recall test on more than 30 volunteers (ages 55–75) before the study, and the two assessors were present at the same time to learn from each other during the evaluation.

2.2. Tool

2.2.1. AVLT operating process

The subjects were told in advance that they would be asked to recall words. The evaluator then read 12 words a second apart. After reading, the subject was required to recall immediately, and the test was conducted three times. The average of the correct words recalled three times was recorded as the "immediate recall" score; 20 min later, the subject was required to recall the words again, and the correct number of words recalled was recorded as the "delayed recall" score. During the 20-min interval, all subjects performed two fixed non-verbal tests, as did the SMOT group.

2.2.2. SMOT improved method and operation process

According to Chinese cultural characteristics, the original cartoon style and picture colors were maintained in the 12 types of object pictures; unlike the original presentation, in our study, the 12 pictures were presented to the subjects in turn rather than altogether. As with the AVLT procedure, subjects were told in advance that they would be asked to recall the pictures. The assessor then presented 12 pictures to each subject in a fixed order and asked the subject to name each picture. Each picture was separated by 1 s, and the exercise was repeated three times. It was not necessary for the subject to give the accurate name of the object shown in the picture; for example, he or she could call a chair a stool, as long as it was evident that the subject understood what the object was. If a subject was unable to name the object immediately, the rater would explain it, and if the subject was still unable to recognize the object during the next two exercises, the rater would explain it again but would not score the picture for either immediate or delayed recall.

2.3. Anesthesia methods and management of surgical patients

Routine ECG monitoring was performed after admission, and sodium lactate Ringer's injection (6 ml/kg) was infused in advance after opening the upper limb venous access. The L3-4 or L2-3 interspace was selected at the puncture site for spinal anesthesia. After the cerebrospinal fluid reflux was unobstructed, 2$\sim$3 ml of heavy 0.5% bupivacaine was slowly injected, and the anesthesia level was adjusted to the T10 level. No sedative drugs were used during the operation,

and the patient was asked to go to the pillow supine position for 6 h after the operation.

2.4. Statistical analysis

SPSS 20.0 software was used for statistical analysis. Measurement data were expressed as the mean ± standard deviation, and enumeration data were expressed as the number of cases and/or rate (%). Independent sample *t*-test, paired sample *t*-test, Chi-square test, multiple linear regression analysis and repeated measures analysis of variance were used for statistical methods. See the section "Results" for specific methods. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Randomization of the study

After screening for inclusion and exclusion criteria, a total of 312 subjects were randomized; after removal, the data of a total of 228 subjects were included in the analysis: the SMOT group ($n = 121$) and AVLT group ($n = 107$), of which the proportion of AVLT that did not finish the test was significantly higher than that of SMOT ($\chi^2 = 10.513$, $P = 0.002$). And the reasons for excluding 84 cases included 36 cases with operation time over 2 h, 28 cases without epidural anesthesia on the day of operation, 8 cases with postoperative pain, and 12 cases with postoperative nausea.

3.2. Comparison between patients and their accompanying family members

There were no significant differences in general data, such as age, sex, education, and two MMSE scores, between surgical patients and their families set as controls except for ASA grade (Table 1).

3.3. General data comparison of the SMOT AVLT group

There was no significant difference in age, sex, identity, education, or other general data between the two groups (Table 2).

3.4. Comparison of SMOT and AVLT first scores

Repeated measures analysis of variance showed that there was a significant difference in the three learning scores between the SMOT and AVLT ($F = 19.249$, $P = 0.000$), so multivariate analysis of variance was used, and the results showed that the three learning scores of the SMOT were significantly higher than those of the AVLT. An independent sample *t*-test showed that the immediate recall and delayed recall scores of the SMOT were significantly higher than those of the AVLT (Table 3).

Immediate recall and delayed recall were similar difficulty in both tests (both close to 0.5), and discrimination was also good (both greater than 0.3) (Table 4). According to the discrimination calculation principle, 27% of scores below the total score were defined as low score, and 73% of scores above the total score were defined as high score; that is, low score for immediate recall was ≤ 9 points and high score was ≥ 27 points; low score for delayed recall was ≤ 3 points and high score was ≥ 9 points. The chi-square test showed that the low scores of immediate recall and delayed recall in AVLT were

TABLE 1 Comparison of general data between patients and their families.

Measures	Patients ($n = 132$)	Controls ($n = 96$)	Statistics	*P*-value
Age, year	63.9 ± 5.3	64.6 ± 5.3	$t = -1.013$	0.311
Sex, female/male	73/59	43/53	$\chi^2 = 3.856$	0.060
Education ≤ primary school/ ≥ junior high school[a]	67/65	51/45	$\chi^2 = 0.377$	0.576
Occupations, category A/category B[b]	64/68	53/43	$\chi^2 = 0.721$	0.423
ASA, I/II	56/76	54/42	$\chi^2 = 4.249$	0.041*
Preoperative MMSE score	24.8 ± 2.2	24.6 ± 2.2	$t = 0.558$	0.574
Postoperative MMSE score	26.0 ± 2.4	26.0 ± 2.3	$t = -0.240$	0.808

[a]Because the proportion of illiterate subjects and those with a high school degree or more was too low, the education level is only divided into two levels: primary school degree or less; junior high school degree or more.
[b]Occupations are classified into two categories according to whether the daily work (before retirement) involved reading, writing, or computer operation: Category A was composed of teachers, doctors, civil servants, etc.; Category B, farmers, housework, caregivers, drivers, etc.
*$p < 0.05$.

TABLE 2 General data comparison of the SMOT AVLT group.

Measures	Group SMOT ($n = 121$)	Group AVLT ($n = 107$)	Statistics	*P*-value
Age, year	63.9 ± 5.7	64.8 ± 5.2	$t = -0.818$	0.411
Sex, female/male	65/56	48/59	$\chi^2 = 1.513$	0.246
Patients/accompanying family members	71/50	64/43	$\chi^2 = 0.000$	1.000
≤ Primary school/ ≥ junior high school	67/54	53/54	$\chi^2 = 1.363$	0.277
Occupations, category A/category B	61/60	55/52	$\chi^2 = 0.169$	0.687
ASA, I/II	59/62	55/52	$\chi^2 = 0.016$	1.000
Preoperative MMSE score	24.8 ± 2.1	24.6 ± 2.3	$t = 0.524$	0.600

TABLE 3 Comparison of the SMOT to AVLT first scores.

Measures	First learn	Second learn	Third learn	Immediate recall	Delayed recall
SMOT ($n = 121$)	5.6 ± 1.7	7.3 ± 2.0	8.6 ± 1.8	21.9 ± 5.0	6.4 ± 1.8
AVLT ($n = 107$)	4.8 ± 1.8	6.6 ± 2.1	7.5 ± 2.1	18.8 ± 5.3	5.6 ± 2.4
Statistics	$F = 12.659$	$F = 10.980$	$F = 22.226$	$t = 4.375$	$t = 4.055$
p-value	0.000*	0.001*	0.000*	0.000*	0.000*

*$p < 0.05$.

TABLE 4 Difficulties, discrimination, and scoring rates between the SMOT and AVLT.

Measures	Immediate recall				Delayed recall			
	Difficulty	Discrimination	Low score end	High score end	Difficulty	Discrimination	Low score end	High score end
SMOT	0.61	0.28	4 (3.3%)	12 (9.9%)	0.57	0.32	7 (5.7%)	10 (8.2%)
AVLT	0.53	0.39	13 (12.1%)	8 (7.4%)	0.46	0.47	21 (19.6%)	8 (7.4%)
Statistics	–	–	$\chi^2 = 8.141$	$\chi^2 = 0.856$	–	–	$\chi^2 = 11.158$	$\chi^2 = 0.049$
p-value	–	–	0.007*	0.471	–	–	0.001*	1.000

Difficulty, that is, the average score rate, is the mean score of a test divided by its full score; after sorting the test scores from high to low, 27% of the data are selected from the two ends to calculate the average of the two ends, respectively (Forrest et al., 1994), and the discrimination is the average score rate of the high score end minus the average score rate of the low score end.
*$p < 0.05$.

TABLE 5 Analysis of test deviation between SMOT and AVLT.

Measures/Results		Age	Identity	Sex	Education	Occupation	Media exposure habits	MMSE
SKT immediate recall	*B*-value	−0.283	−0.669	−0.140	−0.509	0.723	−0.176	0.588
	p-value	0.000*	0.507	0.876	0.543	0.411	0.841	0.003*
AVLT immediate recall	*B*-value	−0.314	0.078	−0.737	1.780	−2.995	−0.032	0.886
	p-value	0.000*	0.913	0.360	0.025*	0.001*	0.982	0.000*
SKT delayed recall	*B*-value	−0.106	0.108	−0.182	0.017	0.407	−0.308	0.091
	p-value	0.000*	0.730	0.554	0.936	0.173	0.319	0.149
AVLT delayed recall	*B*-value	−0.153	−0.283	−0.451	0.313	−0.757	−0.231	0.218
	p-value	0.000*	0.435	0.183	0.359	0.041*	0.551	0.011*

*$p < 0.05$.

significantly higher than those in SMOT, while the high scores were not significantly different (Table 4).

3.5. Analysis of test deviation between SMOT and AVLT

Multiple linear regression analysis with the stepwise method using $\alpha = 0.05$ and $\beta = 0.1$ as dependent variables, age, identity (patient/family member), gender, education, occupation, presence, or absence of media exposure habits, and MMSE basic score as independent variables was performed, and the results showed that age was a significant factor influencing the scores of each test; except SMOT delayed recall, the other tests were closely related to MMSE basic scores; the influencing factors of AVLT test scores were more than SKT tests (Table 5). In addition, there was no significant relationship between the scores of each item of the two tests and the identity of the subjects, further confirming the homogeneity between the family members and the patients.

3.6. Within-group comparison of SMOT and AVLT scores

A paired *t*-test showed that the SMOT second immediate recall score was significantly higher than its first, and there were no significant differences in the two scores on the remaining tests; the high score rate of the SMOT second immediate recall and delayed recall was significantly higher than the first, and there was no significant difference in the scores of the remaining tests (Table 6). In addition, an independent sample *t*-test showed that there were no significant differences in scores between family members and patients in the second test, once again confirming the homogeneity of the two.

4. Discussion

The consensus of multiple international POCD clinical research teams, including ISPOCD, is that multiple neuropsychological test tools must be used in combination for the assessment and diagnosis of POCD (Rasmussen et al., 2001; Hanning, 2005; Rundshagen, 2014). In contrast, in recent years, few researchers have used neuropsychological test batteries in domestic POCD clinical research, and most research teams tend to use comprehensive cognitive assessment scales, of which the MMSE scale is the most widely used (Xiao et al., 2017; Zhang et al., 2017). The MMSE scale is mostly used for dementia screening, and for mild cognitive impairment; its sensitivity and specificity are poor, so most researchers believe that it is not suitable for assessing POCD (Rasmussen et al., 2001; Hanning, 2005; Lin et al., 2013; Rundshagen, 2014). This study also found that there were many problems in the MMSE scale as follows: (1) the difficulty of the first evaluation of MMSE in all subjects was

TABLE 6 Within-group comparison of SMOT and AVLT scores.

Measures		SMOT					AVLT				
		Average score	Difficulty	Discrimination	Low score rate	High score rate	Average score	Difficulty	Discrimination	Low score rate	High score rate
Immediate recall		23.1 ± 5.1	0.65	0.37	2/121	24/121	19.0 ± 6.0	0.53	0.42	10/107	14/107
vs. Preoperative	Statistics	$t = -3.967$	–	–	$\chi^2 = 0.000$	$\chi^2 = 4.669$	$t = -0.829$	–	–	$\chi^2 = 0.443$	$\chi^2 = 3.248$
	p-value	0.000*	–		1.000	0.046	0.413	–	–	0.660	0.117
Delayed recall		6.9 ± 1.8	0.57	0.38	1/121	26/121	5.9 ± 2.2	0.49	0.47	17/107	19/107
vs. Preoperative	Statistics	$t = -1.387$	–	–	$\chi^2 = 4.649$	$\chi^2 = 7.523$	$t = -1.763$	–	–	$\chi^2 = 0.491$	$\chi^2 = 3.671$
	p-value	0.157	–	–	0.061	0.010*	0.088	–	–	0.608	0.079

*$p < 0.05$.

0.83, and the discrimination was 0.16; (2) the learning effect was significant, and the paired *t*-test showed that the second MMSE score in all subjects (26.0 ± 2.4) was significantly higher than the first score (24.7 ± 2.2) ($t = -15.114$, $P = 0.000$); (3) there may be a ceiling effect in the qualitative ability and language ability tests; (4) there may be a floor effect in the attention and calculation tests for subjects with low education levels; (5) the memory evaluation part contained only three words, with too low sensitivity. Therefore, our research team intends to develop a set of neuropsychological test batteries suitable for evaluating POCD in middle-aged and elderly Chinese patients, and this study is one of the research topics in this research direction.

The reasons for selecting patients undergoing elective herniorrhaphy as the observation subjects in this study are as follows: (1) compared with recruiting volunteers, surgical patients are more convenient for follow-up and greatly reduce the loss rate. The total loss rate in this study was 15.1%. All subjects were accompanied by family members. Surgical patients are more likely to cooperate and not likely to give up on taking the test. The 43 subjects who did not complete the test were accompanied by 32 family members. (2) Compared with patients undergoing major surgery and medical inpatients, such patients undergoing elective minor surgery are about the same as the normal population, and this study was strictly limited in the inclusion and exclusion criteria. (3) Based on the purpose of the POCD study, it is convenient to observe the specific factors that may affect cognitive evaluation in surgical patients. For example, this study found that intravenous indwelling has a certain effect on a non-verbal test. The reasons for including family members as subjects in this study are : (1) to facilitate follow-up; (2) expand the sample size to facilitate the study; and (3) serve as a control group to rule out the possible effects of diseases, the inpatient environment, medical intervention and other factors on patients. Study data analysis also confirmed that surgical patients and family volunteers have "homogeneity" in most aspects, especially multiple verifications of cognitive ability. However, there are also some differences, such as the greater proportion of ASA II patients compared to family members, which may be because the medical records of patients are perfect and ASA classification is convenient, while there are more uncertainties in asking the medical history of family members. In addition, although there was no significant difference in the sex ratio between the two groups, there were more males in the family group, which may be attributed to the fact that female patients are mostly accompanied by male family members, while male patients can sign the anesthesia informed consent by themselves.

AVLT is one of the three neuropsychological test methods recommended by the 1995 Consensus Conference on Cognitive Impairment Assessment after Cardiac Surgery (Murkin et al., 1995; Xiao et al., 2017; Zhang et al., 2017) and is also used by many international POCD clinical research teams in addition to ISPOCD (Silbert et al., 2014, 2015). In this study, we used the Huashan version prepared by Professor Guo Qihao, which has confirmed the validity and reliability of AVLT in research fields such as mild cognitive impairment (MCI) (Guo et al., 2007; Zhao et al., 2015) and is used by many researchers in China (Liu et al., 2012; Li et al., 2016). Since our team found that AVLT poses many problems for the elderly population with a low level of education level, we developed a modified version of the SMOT based on the SKT memory subtest. To prepare the SMOT, we designed a total of 20 pictures. After data analysis of 50 subjects aged 55–75 years, the 8 pictures with the

lowest identification were removed, and the remaining 12 pictures had 100% identification. In this study, all subjects said the correct name of each object. In this study, we found that the scores of all items of SMOT were significantly higher than those of AVLT, and their difficulty and discrimination were not significantly different from those of AVLT. In addition, according to the principle of discrimination, this study defined "low score" and "high score" to analyze the potential ceiling and floor effects of the test, and the results showed that there was no significant difference in the high score rate between the two tests, but the low score of immediate recall and delayed recall of AVLT was significantly lower than that of SMOT. Further analysis found that approximately 80% of the low score of AVLT was still the low score in the second test, indicating that AVLT was likely to have floor effect on this part of subjects. The test deviation analysis showed that the influencing factors of AVLT were greater than those of SMOT; that is, AVLT had poor general applicability to the population aged 50–70 years, and there was interference in the diagnosis of cognitive impairment. For example, occupation was the influencing factor of AVLT. If there were differences in the proportion of normal occupations, errors would occur when the Z score was calculated with the mean and standard deviation of the normal group to offset the learning effect in the diagnosis of POCD by the *Z*-score. However, the within-group comparison revealed that SMOT had a significant learning effect on immediate recall, and although the test interval was 2 days in this study, although AVLT had no significant learning effect, the low score rate on the second test was still high, and 17 of the 18 patients with low scores on delayed recall also had low scores on the first test, further indicating that AVLT may have a floor effect (Beier et al., 2019; Holmgaard et al., 2019; McGovern et al., 2019).

The subjects observed in this study were middle-aged and elderly populations aged 55–75 years. Because this age group has a high degree of cooperation with difficult neuropsychological tests and has better clinical preventive significance for POCD, it is also the main research target population of our POCD clinical research team. However, from the perspective of norms, the number of observations in this study is still small, and there are regional restrictions. In addition, although the SMOT and AVLT are objective neuropsychological tests, the rater reliability analysis is conducive to ruling out the interference of assessor-related factors. However, this study did not analyze them but made sufficient pretest preparation to ensure the consistency of assessor reliability.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

LC contributed to write the article, critical revision of the article, and statistical analysis. BG performed critical revision of the article, final approval of the article, and statistical analysis. CY performed the data collection and critical revision of the article. HJ performed conception and design, wrote the article, and critical revision of the article. ZW helped perform the data collection and critical revision of the article. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

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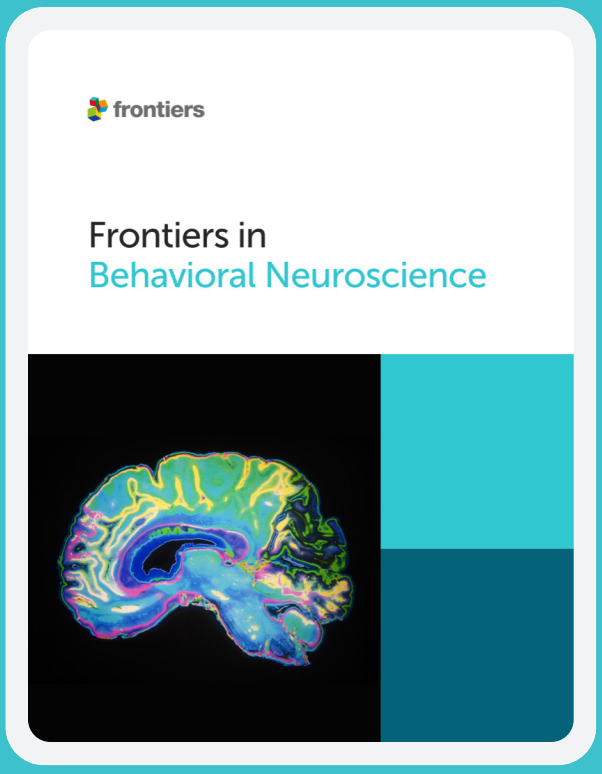

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