Behavioural Brain Research 408 (2021) 113283

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Activation of cholinergic system partially rescues olfactory dysfunction-induced learning and memory deficit in mice

Kohei Takahashi [a], Minoru Tsuji [a,*], Osamu Nakagawasai [b], Soh Katsuyama [c], Kazuya Miyagawa [a], Kazuhiro Kurokawa [a], Atsumi Mochida-Saito [a], Masahiro Iwasa [d], Hiroyuki Iwasa [d], Hiroshi Takeda [e], Takeshi Tadano [b,f]

[a] Department of Pharmacology, School of Pharmacy, International University of Health and Welfare, 2600-1 Kitakanemaru, Ohtawara, Tochigi 324-8501, Japan
[b] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, Japan
[c] Division of Clinical Pharmacology and Pharmaceutics, Nihon Pharmaceutical University, 10281 Komuro, Kitaadachigun Inamachi, Saitama 362-0806, Japan
[d] Nihon Berm Co., Ltd, 2-14-3 Nagatachou, Chiyoda-ku, Tokyo 100-0014, Japan
[e] Department of Pharmacology, School of Pharmacy at Fukuoka, International University of Health and Welfare, 137-1 Enokizu, Okawa, Fukuoka 831-8501, Japan
[f] Complementary and Alternative Medicine Clinical Research and Development, Graduate School of Medicine Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8640, Japan

ARTICLE INFO

Keywords:
Choline acetyltransferase
Hippocampus
Memory
Olfactory function
Zinc sulfate

ABSTRACT

Deficits in olfaction are associated with neurodegenerative disorders such as Alzheimer's disease. A recent study reported that intranasal zinc sulfate ($ZnSO_4$)-treated mice show olfaction and memory deficits. However, it remains unknown whether olfaction deficit-induced learning and memory impairment is associated with the cholinergic system in the brain. In this study, we evaluated olfactory function by the buried food find test, and learning and memory function by the Y-maze and passive avoidance tests in $ZnSO_4$-treated mice. The expression of choline acetyltransferase (ChAT) protein in the olfactory bulb (OB), prefrontal cortex, hippocampus, and amygdala was assessed by western blotting. Moreover, we observed the effect of the acetylcholinesterase inhibitor physostigmine on $ZnSO_4$-induced learning and memory deficits. We found that intranasal $ZnSO_4$-treated mice exhibited olfactory dysfunction, while this change was recovered on day 14 after treatment. Both short-term and long-term learning and memory were impaired on days 4 and 7 after treatment with $ZnSO_4$, whereas the former, but not the latter, was recovered on day 14 after treatment. A significant correlation was observed between olfactory function and short-term memory, but not long-term memory. Treatment with $ZnSO_4$ decreased the ChAT level in the OB on day 4, and increased and decreased the ChAT levels in the OB and hippocampus on day 7, respectively. Physostigmine improved the $ZnSO_4$-induced deficit in short-term, but not long-term, memory. Taken together, the present results suggest that short-term memory may be closely associated with olfactory function via the cholinergic system.

1. Introduction

Olfaction plays an important role in several behaviors such as food localization, emotional modulation, and social behaviors. Odors are first detected by olfactory sensory neurons in the olfactory epithelium, and this information is then transferred to the olfactory bulb (OB) which projects to brain areas such as the cortex, hippocampus and amygdala, which play crucial roles in cognition [1].

It is well known that Alzheimer's disease (AD) patients show marked olfactory dysfunction [2], and this change is observed in an early stage of AD [3]. AD patients show a marked decrease in choline acetyltransferase (ChAT) activity, which is responsible for the synthesis of the transmitter acetylcholine in the OB and its projection to brain areas such as the cortex, hippocampus, and amygdala [4–7]. Cholinergic neurons in

Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; ChAT, choline acetyltransferase; i.p., intraperitoneally; LTP, long-term potentiation; OB, olfactory bulb; OBX, olfactory bulbectomy; PFC, prefrontal cortex; SEM, standard error of the mean; SVZ, subventricular zone; TBST, tris-buffered saline supplemented with 0.01 % Tween-20; $ZnSO_4$, zinc sulfate.

* Corresponding author.
E-mail address: mtsuji@iuhw.ac.jp (M. Tsuji).

https://doi.org/10.1016/j.bbr.2021.113283
Received 1 December 2020; Received in revised form 18 March 2021; Accepted 30 March 2021
Available online 2 April 2021

the brain play crucial roles in olfactory and memory function [8]. Anticholinergic drugs such as atropine and scopolamine cause olfactory and memory deficits [9–14], while acetylcholinesterase inhibitors such as donepezil recover these deficits [13,15,16]. These findings suggest that changes in the olfactory system and cholinergic neurons may be associated with AD symptoms.

Olfactory bulbectomy (OBX) can lead to various abnormal behaviors such as depressive-like behavior [17–20] and cognitive dysfunction [21–23], as well as various neurochemical changes such as reductions in monoamines [19,24,25], hippocampal neurogenesis [17,19,22,23], long-term potentiation (LTP) [26], and choline acetyltransferase (ChAT) levels [21]. However, OBX results in permanent anosmia, damages blood circulation in the brain [27], and leads to retrograde neurodegeneration of neurons in the cortex, hippocampus, amygdala and more [28,29]. Thus, this model in rodents appears to be unsuitable for evaluating the association between olfaction and memory function.

Nasal administration of zinc sulfate ($ZnSO_4$) has been shown to result in reversible lesion of the olfactory epithelium and transient anosmia in mice [30]. Recently, other studies have reported that nasal treatment with $ZnSO_4$ induces memory and learning impairment, and a reduction of hippocampal LTP in mice [31,32]. Hence, this model is appropriate for evaluating the impact of olfactory dysfunction on learning and memory. However, it remains unknown whether $ZnSO_4$-induced cognitive dysfunction is associated with the cholinergic system in the brain.

In the present study, memory function after nasal treatment with $ZnSO_4$ was examined in the Y-maze and passive avoidance tests to examine whether olfactory dysfunction affects learning and memory function. Moreover, to evaluate whether olfactory dysfunction-induced memory deficits are associated with cholinergic neurons, we investigated the changes in ChAT levels in the brain regions of mice treated with $ZnSO_4$ and the effect of the acetylcholinesterase inhibitor physostigmine on $ZnSO_4$-induced memory deficits.

2. Materials and methods

All experiments were approved by the Ethics Committee of Animal Experiments at the International University of Health and Welfare (Ohtawara, Japan; approval number: 19016). All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD). Efforts were made to minimize the animals' suffering and reduce the number of animals used.

2.1. Animals

We used male ddY mice (age, 6–7 weeks; weight, 26–28 g; Japan SLC, Shizuoka, Japan) for all experiments (total, n = 427; behavioral test, n = 367; western blotting analysis, n = 60). The mice were housed in cages containing five to six mice, and subjected to steady conditions (i.e., temperature, 23 ± 1 °C; humidity, 55 ± 5 %, and 12/12 h light-dark cycle with lights on at 7:00). All behavioral tests were performed between 10:00 and 17:00. Each animal was tested for only one behavior except for the buried food finding test and the evaluation of the relationship between olfaction and memory-related behaviors. The behavioral tests were conducted by a blinded observer.

2.2. Olfactory deprivation

Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Dainippon, Osaka, Japan), and then subjected to intranasal $ZnSO_4$ or saline effusion in an abdomen-up position. Ten μl of saline or 2.5 % $ZnSO_4$ (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) solution was intranasally perfused into each nostril with a 10 μl pipette (total 20 μl per mouse).

2.3. Drugs and treatments

Physostigmine (Sigma-Aldrich, St-Louis, MO, USA) was dissolved in saline. Physostigmine (0.03 and 0.1 mg/kg) was intraperitoneally (i.p.) administered 30 min before the behavioral tests on day 7 after nasal administration of saline or $ZnSO_4$. These drugs were administered in a volume of 0.1 mL/10 g of mouse body weight. The dose for each drug used was calculated from previous reports [21].

2.4. Buried food finding test

The buried food finding test was used as a measure of olfactory function in mice. Briefly, mice were housed singly in a cage (height: 17 cm, width: 25 cm, length: 30 cm) with clean 3 cm-deep bedding for 5 min after 24 h of food fasting. Food was randomly buried 0.5 cm under the bedding surface in the cage. The latency to find the food was recorded manually. This test was conducted on days 0 (pretreatment), 4, 7, and 14 after the administration of $ZnSO_4$.

2.5. Y-maze test

The Y-maze apparatus consisted of three compartments (height: 25 cm, width: 3 cm, length: 40 cm) radiating out from the center. The mice were placed in one of the compartments and allowed to move freely for 8 min. Experiments were performed at a light intensity of 35 lx. An arm entry was defined as three legs entering one of the arms, and the sequence of entries was manually recorded. An alternation was defined as entry into all three arms on consecutive trials. Thus, the maximum number of alternations was the total number of entries minus 2, and the percent alternation was calculated as (actual alternations / maximum alternations) × 100. The percent spontaneous alternation behavior of the mouse was taken as a measure of spatial short-term memory.

2.6. Passive avoidance test

The passive avoidance test was performed as previously described [33]. In the training trial, an electric foot-shock (0.5 mA for 1 s) was delivered to the feet through the floor grids as soon as the mouse left the lighted compartment and completely entered the dark compartment. This trial was conducted 24 h after nasal administration of saline or $ZnSO_4$. In the test trial, each mouse for each time point was placed in the illuminated compartment and the latency to enter the dark compartment was once again recorded, allowing a maximum cut-off time of 300 s; no foot-shock was delivered in this case.

2.7. Western blotting

To confirm the expression of ChAT in the OB, prefrontal cortex (PFC), hippocampus, and amygdala of mice on day 4, 7, or 14 after nasal administration of saline or $ZnSO_4$, mice were sacrificed by decapitation; the brain was immediately removed and the OB, PFC, hippocampus, and amygdala were dissected quickly by a mouse brain slicer (Muromachi Kikai). We used samples from mice that had not undergone any behavioral tests. The brain atlas of Paxinos and Franklin [34] was used as a reference to guide all dissections. Protein isolation and western blotting were performed as described previously [20]. After electrophoresis, proteins were transferred electrically from the gel onto a polyvinylidene difluoride membrane using a semi-dry blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The blots were blocked for 30 min with 5 % skim-milk in Tris-buffered saline supplemented with 0.01 % Tween-20 (TBST). Next, membranes were probed with antibodies against ChAT (1:1000; Millipore Corporation, Billerica, MA, USA) and β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The blots were washed several times and then incubated at room temperature for 1 h with secondary antibody

(horseradish peroxidase-COnjugated anti-goat or anti-mouse IgG antibody diluted 1:10000 with TBST containing 5 % skim-milk). Blots were developed using an ImmunoCruz (Santa Cruz Biotechnology) and scanned, optimized and analyzed by Quantity One 1-D Analysis Software version 4.5.2 (Bio-Rad Laboratories). The density of the corresponding bands was analyzed using Image Studio Lite version 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

2.8. Statistical analysis

Results of experiments are expressed as mean ± standard error of the mean (SEM). Normality and homoscedasticity assumptions were verified prior to the use of any parametric tests (Shapiro-Wilk normality test and equality of variances F-test). The statistical significance of differences was determined by the Student's *t*-test for two-group comparisons. The significance of differences was determined by one- or two-way analysis of variance (ANOVA), followed by the Tukey-Kramer test for multiple group comparisons except for the buried food finding test, which was analyzed using the Dunnett test. The criterion of significance was set at $p < 0.05$.

3. Results

3.1. Change in olfactory function after the nasal administration of $ZnSO_4$ in mice

As shown in Fig. 1, nasal administration of 2.5 % $ZnSO_4$ induced a significant increase in the time required to find the buried food on days 4 and 7 after treatment (pre vs day 4: $p = 0.0005$; pre vs day 7: $p = 0.0252$), and this change recovered on day 14 after treatment with $ZnSO_4$ (day 4 vs day 14: $p = 0.0010$) [One-way ANOVA: $F(3, 68) = 7.916$, $p = 0.0001$]. In addition, the time required to find the buried food on day 14 after nasal administration of $ZnSO_4$ was not significantly different from that of pretreatment group (pre vs day 14: $p = 0.2758$). We observed that 5 % $ZnSO_4$-treated mice almost all died immediately after administration (data not shown). Hence, we considered that 2.5 % $ZnSO_4$ was the appropriate dose to investigate the relationship between olfaction and memory.

3.2. Time-dependent effect of $ZnSO_4$ on learning and memory in mice

A two-way ANOVA revealed a significant interaction between time and group for spontaneous alternation behavior [$F(2, 84) = 5.493$, $p = 0.0057$, Fig. 2(B)], but not for number of arms [$F(2, 84) = 0.6969$, $p = 0.5010$, Fig. 2(C)]. $ZnSO_4$-treated mice showed a decrease in spontaneous alternation behavior on day 4 or 7 after nasal administration compared to each saline group (day 4: $p = 0.0010$; day 7: $p < 0.0001$), while this deficit was not observed on day 14 (day 4 vs day 14: $p = 0.0011$). There was no significant difference in total arm entries between saline and $ZnSO_4$-treated mice for any of the periods.

A two-way ANOVA revealed a significant interaction between time and group for latency time in the passive avoidance test [$F(3, 112) = 5.675$, $p = 0.0012$, Fig. 3(B); $F(1, 38) = 9.007$, $p = 0.0047$, Fig. 3(D)]. On day 1 (training trial day), all groups showed similar latency times to enter the dark compartment [day 1: $p > 0.9999$, Fig. 3(B)]. $ZnSO_4$-treated mice had a significantly lower latency time compared to the saline group on days 4, 7, and 14 after treatment [Fig. 3(B): day 4: $p = 0.0007$; day 7: $p = 0.0240$; day 14: $p < 0.0001$, Fig. 3(B)]. Moreover, we examined learning and memory function when $ZnSO_4$-treated mice had mostly recovered from anosmia. On day 14 (training trial day), all groups showed similar latency times to enter the dark compartment [day 14: $p > 0.9999$, Fig. 3(D)], while $ZnSO_4$-treated mice had a significantly lower latency time compared to the saline group on day 17 after treatment [day 17: $p = 0.0008$, Fig. 3(D)].

3.3. Correlations of olfactory function with memory-related behavior in $ZnSO_4$-treated mice

Using linear regression, we examined the relationship between olfactory function and memory-related behavior on day 7 after treatment with $ZnSO_4$. We found a significant correlation between the latency time to find the buried food and the spontaneous alternation behavior in the Y-maze test [$R^2 = 0.3393$, $r = -0.5825$, $p = 0.0179$, Fig. 4(A)], but not the latency time in the passive avoidance test [$R^2 = 0.04045$, $r = -0.2011$, $p = 0.4551$, Fig. 4(B)].

3.4. Changes in ChAT levels in brain regions after $ZnSO_4$ treatment

A two-way ANOVA revealed a significant interaction between time and group for the OB and hippocampus [$F(2, 54) = 11.47$, $p < 0.0001$, Fig. 5(A); $F(2, 54) = 4.598$, $p = 0.0143$, Fig. 5(C)], but not for the PFC or amygdala [$F(2, 54) = 0.07558$, $p = 0.9273$, Fig. 5(B); $F(2, 54) = 1.587$, $p = 0.2139$, Fig. 5(D)]. On day 4 after administration, $ZnSO_4$-treated mice exhibited a significant decrease in ChAT levels in the OB compared to the saline group [$p = 0.0155$, Fig. 5(A)]. On day 7 after administration, $ZnSO_4$-treated mice exhibited a significant increase and decrease in ChAT levels in the OB and hippocampus, respectively [OB: $p = 0.0020$, Fig. 5(A); hippocampus: $p = 0.0028$, Fig. 5(C)]. On day 14 after treatment, the ChAT levels in the OB and hippocampus were unchanged between the saline and $ZnSO_4$-treatment groups [OB: $p = 0.1827$, Fig. 5

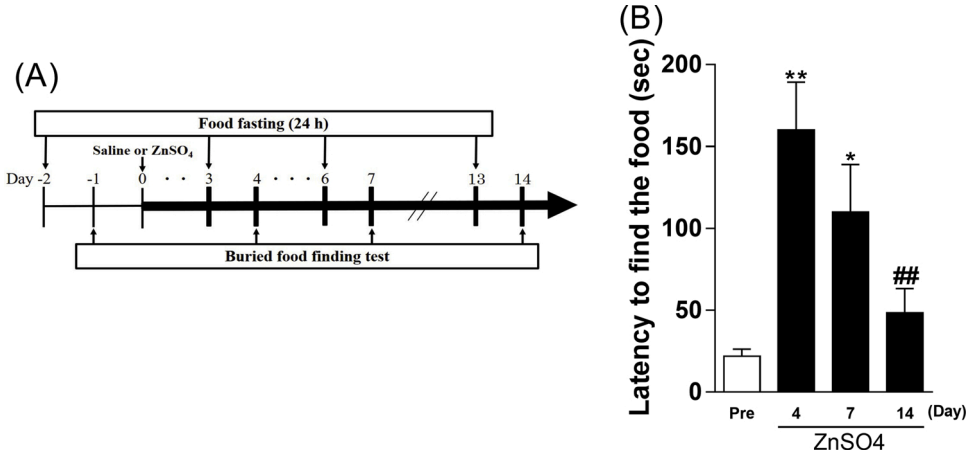

Fig. 1. Nasal administration of $ZnSO_4$ induces reversible olfactory dysfunction in mice. A: Time course of the experimental protocol. B: Latency times to find the buried food during the buried food finding test in mice. Bars represent means ± SEM. * $p < 0.05$ and ** $p < 0.01$ vs. pretreatment group. ## $p < 0.01$ vs. day 4 after $ZnSO_4$ treatment group ($n = 18$).

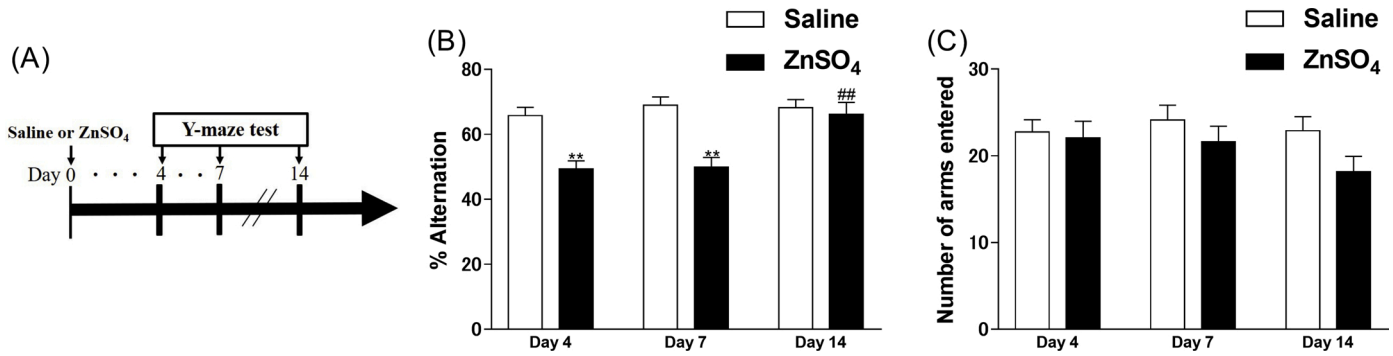

Fig. 2. $ZnSO_4$-treated mice show short-term memory impairment in the Y-maze test. A: Time course of the experimental protocol. B and C: Spontaneous alternation behavior (B) and locomotor activity (C) during the Y-maze test in mice. Bars represent means ± SEM. ** $p < 0.01$ vs. saline group. ## $p < 0.01$ vs. day 4 after $ZnSO_4$ treatment group (n = 13–17 per group).

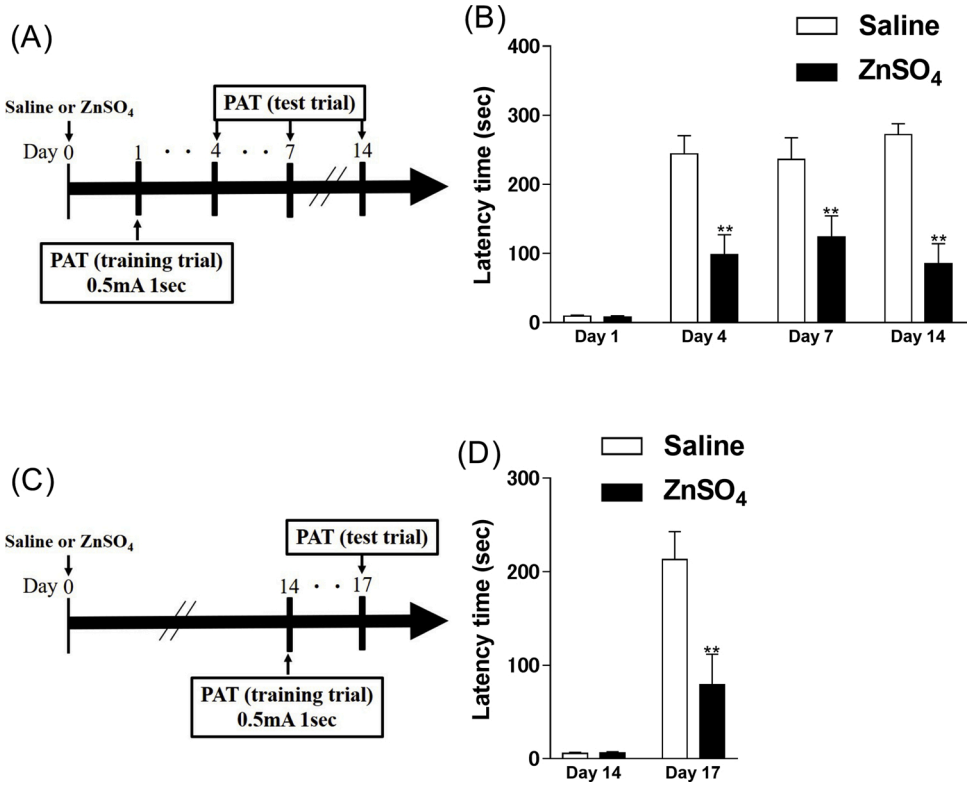

Fig. 3. $ZnSO_4$-treated mice show long-term memory impairment in the passive avoidance test. A and C: The time course of the experimental protocol. B and D: The latency times for the different groups are shown for the training trial at day 1 (B) or day 14 (D) after treatment with $ZnSO_4$ and the test trial in the passive avoidance test. Bars represent means ± SEM. ** $p < 0.01$ vs. saline group (n = 15 per group).

(A); hippocampus: $p = 0.2352$, Fig. 5(C)].

3.5. *Effects of physostigmine on $ZnSO_4$-induced learning and memory deficits in mice*

On day 7 after administration, $ZnSO_4$-treated mice exhibited significant decreases in spontaneous alternation behavior in the Y-maze test ($p = 0.0081$) and in latency time in the passive avoidance test ($p = 0.0089$). Physostigmine (0.1 mg/kg) reversed the decrease in spontaneous alternation behavior ($p = 0.0130$) [one-way ANOVA: $F(3, 55) = 3.687$, $p = 0.0172$, Fig. 6(A)], but had no effect on the decrease in latency time ($p = 0.4963$) [one-way ANOVA: $F(3, 55) = 3.687$, $p = 0.0172$, Fig. 6(C)]. Moreover, locomotor activity as assessed by total arm entries in the Y-maze test was not significantly altered by the administration of physostigmine [one-way ANOVA: $F(3, 53) = 1.381$, $p = 0.2586$, Fig. 6(B)].

4. Discussion

In this study, olfactory dysfunction was time-dependently associated with learning and memory, and with changes in ChAT levels in several brain regions. On days 4 and 7 after intranasal $ZnSO_4$ treatment, mice exhibited complete anosmia, along with memory impairment in the Y-maze and passive avoidance tests, which are used to evaluate short- and long-term memory, respectively. On day 14 after treatment, olfaction and short-term memory deficits recovered, while long-term memory was still impaired. On day 4 after treatment, $ZnSO_4$-treated mice exhibited a significant decrease in ChAT levels in the OB. Moreover, $ZnSO_4$-treated mice exhibited a significant increase in ChAT levels in the OB, and a decrease in ChAT levels in the hippocampus on day 7 after treatment.

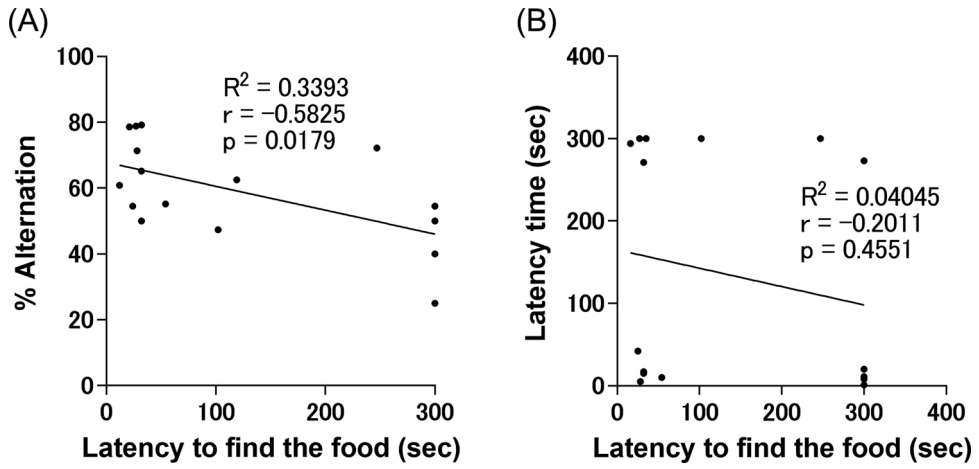

Fig. 4. Linear regression analysis reveals significant correlations between spontaneous alternation behavior, but not latency time, and olfactory function on day 7 after $ZnSO_4$ treatment. The relationship between spontaneous alternation behavior (A) or latency time (B) and olfactory function (n = 16).

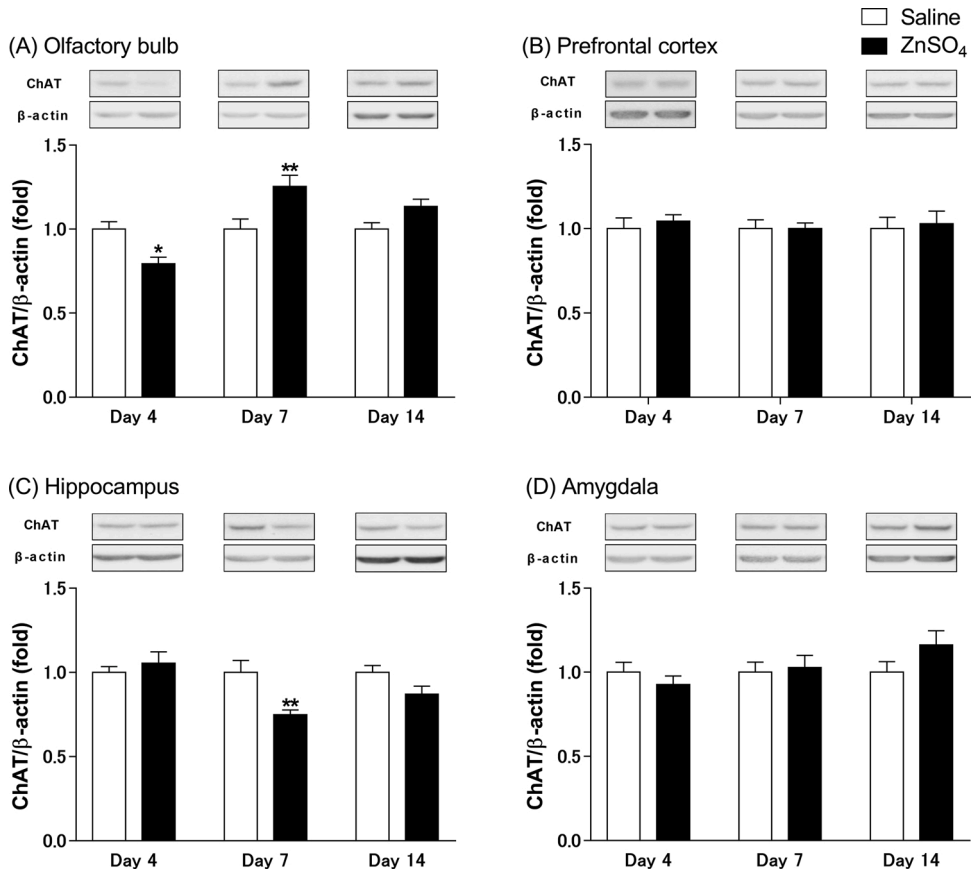

Fig. 5. Changes in the levels of ChAT in the OB (A), PFC (B), hippocampus (C), and amygdala (D) of $ZnSO_4$-treated mice. Bars represent means ± SEM. * $p < 0.05$, ** $p < 0.01$ vs. saline group. (n = 10 per group).

Olfactory function could be destroyed by $ZnSO_4$ as early as 1 h after treatment and persisted for about 1 week [35,36], whereas this dysfunction would eventually disappear because of the recovery of olfactory epithelium cells [35]. Ahn et al. reported that $ZnSO_4$-treated mice showed time-dependent recovery of olfactory epithelium along with the improvement of olfaction [30]. Hence, we consider that change in olfaction closely depends on the degree of olfactory epithelial destruction in $ZnSO_4$-treated mice. In the present study, we also observed that $ZnSO_4$-induced olfactory dysfunction persisted for 1 week, while this change recovered on day 14 after $ZnSO_4$ treatment (Fig. 1). This finding suggests the presence of reversible impaired olfaction in $ZnSO_4$-treated mice.

Olfaction plays crucial roles in cognition [1]. A previous study showed that nasal administration of $ZnSO_4$ induced olfactory dysfunction and changes in learning and memory functions in mice [32]. The present study revealed that $ZnSO_4$-treated mice showed short- and long-term memory impairment on days 4 and 7 after nasal treatment, while short-term memory, but not long-term memory, was recovered accompanied by a return of olfaction on day 14 after nasal treatment (Figs. 2 and 3). Moreover, we found that $ZnSO_4$-treated mice showed

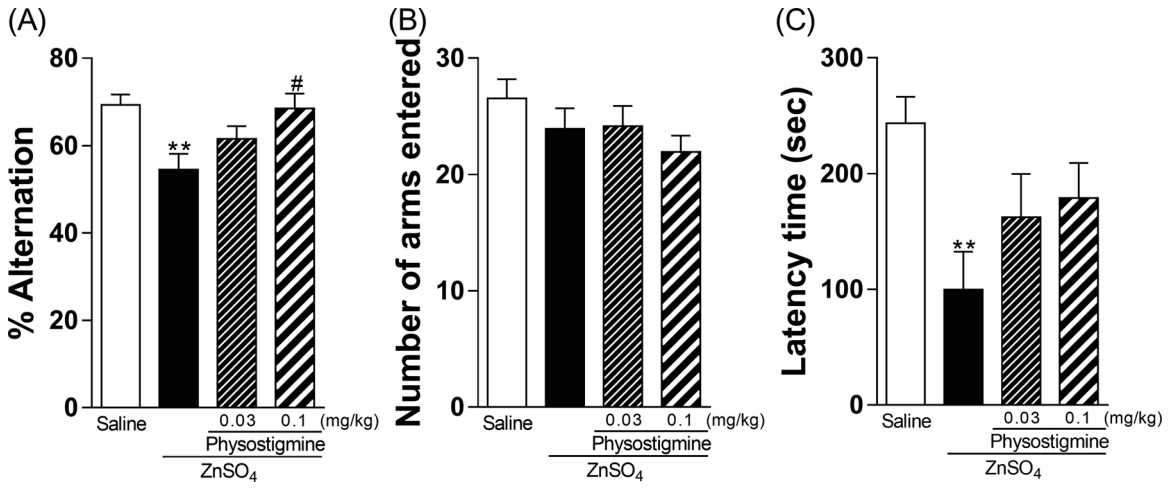

Fig. 6. Physostigmine improves $ZnSO_4$-induced short-term, but not long-term, memory deficit. A and B: Spontaneous alternation behavior (A) and locomotor activity (B) during the Y-maze test in mice. C: The latency times for the different groups are shown for the test trial in the passive avoidance test. Bars represent means ± SEM. ** $p < 0.01$ vs. saline + vehicle group. # $p < 0.05$ vs. $ZnSO_4$ + vehicle group (n = 13–15 per group).

impaired learning function on day 14 after treatment, even though olfactory function was mostly restored [Fig. 3(D)]. Another study reported that associative learning and memory, as well as hippocampal LTP, remain impaired when olfactory function is mostly recovered [31]. The Y-maze test evaluates short-term memory, while the passive avoidance test evaluates associative learning and memory [37]. Thus, we consider that olfactory dysfunction may reversibly reduce short-term memory and induce irreversible associative learning and memory deficits. Furthermore, the present study revealed a significant association between the latency to find buried food and spontaneous alternation behavior in the Y-maze test, but not latency time in the passive avoidance test [Fig. 4(A) and (B)]. These results suggest that olfactory dysfunction may be closely associated with short-term memory.

Muscarinic acetylcholine receptors play a prominent role in the olfactory pathway [38–41]. Interestingly, intranasal administration of atropine, a muscarinic antagonist, is associated with olfactory dysfunction in patients at high risk of developing AD [15,40]. Muscarinic cholinergic transmission plays a major role in odor identification, and there is initial evidence that improvement in odor identification is associated with clinical improvement in patients with AD who receive cholinesterase inhibitors [38]. The activity of ChAT in the olfactory tubercle is known to be reduced in AD patients, indicating that cholinergic neurons may be degenerated in the olfactory system [4]. In the present study, $ZnSO_4$-treated mice showed a decrease in the ChAT level in the OB on day 4 and an increase on day 7 after treatment [Fig. 5(A)]. These changes may reflect reversible olfactory dysfunction. This study demonstrated that olfaction deficits persisted for 1 week (Fig. 1). The reduction of ChAT in the OB on day 4 after treatment may be related to olfactory dysfunction by $ZnSO_4$. In contrast, the increase in ChAT in the OB on day 7 after treatment may contribute to the process of recovery from anosmia. Therefore, it is suggested that time-dependent changes in the ChAT level in the OB may be associated with olfactory changes in $ZnSO_4$-treated mice.

Hippocampal acetylcholine is associated with learning and memory function [42–44]. Infusion of the anticholinergic drug scopolamine into the hippocampus impairs spatial memory in rodents [9–11]. AD patients show a marked decrease in hippocampal ChAT activity [4], and drugs that increase endogenous acetylcholine, such as donepezil and rivastigmine, are used to treat dementia. These reports suggest that modulation of the cholinergic system in the hippocampus plays an important role in memory function. In the present study, we found that $ZnSO_4$-treated mice showed a decrease in the ChAT level in the hippocampus [Fig. 5(C)], and the $ZnSO_4$-induced short-term memory deficit was improved by acute administration of physostigmine on day 7 after treatment [Fig. 6(A)]. Acute treatment with physostigmine also tended to improve $ZnSO_4$-induced long-term memory deficit in the passive avoidance test, but this effect was not statistically significant [Fig. 6(C)]. In contrast, $ZnSO_4$-treated mice showed impaired short-term memory even on day 4 after treatment, despite the unchanged ChAT level in the hippocampus. These results suggest that activation of cholinergic neurons may be associated with the improvement of $ZnSO_4$-induced short-term memory deficit, but the reduction of ChAT in the hippocampus may not directly contribute to the development of $ZnSO_4$-induced short-term memory deficit. In addition, although the ChAT levels in the brain were unchanged, $ZnSO_4$-treated mice showed a long-term memory deficit on day 14 after treatment. An et al. reported that $ZnSO_4$-treated mice exhibited impaired associative learning and memory with decreased hippocampal LTP when olfactory function was mostly recovered [31]. Moreover, recent study reported that hippocampal dopaminergic and glutaminergic systems involved in the induction of LTP were downregulated in $ZnSO_4$-treated mice [45]. Thus, we considered that $ZnSO_4$-induced long-term memory deficit may be associated with, at least in part, the impairment of hippocampal LTP via dysfunction of dopaminergic and/or glutaminergic systems rather than cholinergic system.

The present study revealed that $ZnSO_4$-induced olfactory deficit affects learning and memory functions in mice, but the mechanism remains unclear. Previous studies have suggested that neurogenesis in the subventricular zone (SVZ) is associated with both olfactory function and memory [46,47]. Therefore, we plan to perform additional experiments focusing on neurogenesis in the SVZ in a future study.

In conclusion, the present study demonstrated that nasal treatment with $ZnSO_4$ causes reversible olfactory dysfunction and short-term memory impairment, as well as irreversible long-term memory impairment in mice. These reversible phenomena may contribute to the changes in ChAT levels in the OB and hippocampus by $ZnSO_4$. Moreover, $ZnSO_4$-induced short-term memory impairment was improved by acute administration of physostigmine. The present findings suggest that short-term memory may be closely associated with olfactory function via the cholinergic system, which should contribute to our understanding of the pathophysiology of cognitive disorders such as AD. However, as it remains unknown whether the rescue effects of physostigmine for short-term memory deficits are actually mediated by increased acetylcholine at the OB and hippocampus, we will examine these issues in a future study.

Funding sources

This study was supported, in part, by a Grant-in-Aid for Scientific Research [grant number 19K23808].

CRediT authorship contribution statement

Kohei Takahashi: Validation, Methodology, Conceptualization, Writing - original draft, Formal analysis, Funding acquisition. **Minoru Tsuji:** Writing - review & editing. **Osamu Nakagawasai:** Writing - review & editing, Methodology, Investigation. **Soh Katsuyama:** Methodology, Investigation. **Kazuya Miyagawa:** Investigation. **Kazuhiro Kurokawa:** Investigation. **Atsumi Mochida-Saito:** Investigation. **Masahiro Iwasa:** Supervision. **Hiroyuki Iwasa:** Supervision. **Hiroshi Takeda:** Supervision. **Takeshi Tadano:** Conceptualization, Supervision, Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

The authors would like to thank Mr. Shota Sekii, Mr. Masahiro Kimura, Ms. Mio Koiwa, Ms. Saki Yasuda, and Ms. Nagisa Horino of the International University of Health and Welfare (Ohtawara, Japan) for their technical assistance.

References

[1] Y. Soudry, C. Lemogne, D. Malinvaud, S.M. Consoli, P. Bonfils, Olfactory system and emotion: common substrates, Eur. Ann. Otorhinolaryngol. Head Neck Dis. 128 (1) (2011) 18–23.
[2] M.D. Warner, C.A. Peabody, J.J. Flattery, J.R. Tinklenberg, Olfactory deficits and Alzheimer's disease, Biol. Psychiatry 21 (1) (1986) 116–118.
[3] T. Kovács, N.J. Cairns, P.L. Lantos, Olfactory centres in Alzheimer's disease: olfactory bulb is involved in early Braak's stages, Neuroreport 12 (2) (2001) 285–288.
[4] J. Simpson, C.M. Yates, A. Gordon, D.M. St Clair, Olfactory tubercle choline acetyltransferase activity in Alzheimer-type dementia, Down's syndrome and Huntington's chorea, J. Neurol. Neurosurg. Psychiatry 47 (10) (1984) 1138–1139.
[5] M.N. Rossor, P.C. Emson, C.Q. Mountjoy, M. Roth, L.L. Iversen, Reduced amounts of immunoreactive somatostatin in the temporal cortex in senile dementia of Alzheimer type, Neurosci. Lett. 20 (3) (1980) 373–377.
[6] T.D. Bird, S. Stranahan, S.M. Sumi, M. Raskind, Alzheimer's disease: choline acetyltransferase activity in brain tissue from clinical and pathological subgroups, Ann. Neurol. 14 (3) (1983) 284–293.
[7] E.K. Perry, M. Johnson, A. Ekonomou, R.H. Perry, C. Ballard, J. Attems, Neurogenic abnormalities in Alzheimer's disease differ between stages of neurogenesis and are partly related to cholinergic pathology, Neurobiol. Dis. 47 (2) (2012) 155–162.
[8] M.E. Hasselmo, The role of acetylcholine in learning and memory, Curr. Opin. Neurobiol. 16 (6) (2006) 710–715.
[9] A. Blokland, W. Honig, W.G. Raaijmakers, Effects of intra-hippocampal scopolamine injections in a repeated spatial acquisition task in the rat, Psychopharmacology (Berl.) 109 (3) (1992) 373–376.
[10] J.L. Rogers, R.P. Kesner, Cholinergic modulation of the hippocampus during encoding and retrieval, Neurobiol. Learn. Mem. 80 (3) (2003) 332–342.
[11] W. Chen, X. Cheng, J. Chen, X. Yi, D. Nie, X. Sun, J. Qin, M. Tian, G. Jin, X. Zhang, Lycium barbarum polysaccharides prevent memory and neurogenesis impairments in scopolamine-treated rats, PLoS One 9 (2) (2014), e88076.
[12] M. Serby, C. Flicker, B. Rypma, S. Weber, J.P. Rotrosen, S.H. Ferris, Scopolamine and olfactory function, Biol. Psychiatry 28 (1) (1990) 79–82.
[13] D.P. Devanand, X. Liu, R.E. Chunga, H. Cohen, H. Andrews, P.W. Schofield, Y. Stern, E.D. Huey, J. Choi, G.H. Pelton, Odor identification impairment and change with cholinesterase inhibitor treatment in mild cognitive impairment, J. Alzheimer's Dis. 75 (3) (2020) 845–854.
[14] S. Rafiq, S. Ahmad, F. Ahmed, Z. Batool, S.B. Ahmed, S. Saleem, F. Naqvi, L. Liaquat, A. Afzal, S. Haider, Anticholinergic drug atropine diminishes newly formed fear memory in male rats, Pak. J. Pharm. Sci. 31 (3(Supplementary)) (2018) 1075–1079.
[15] D.P. Devanand, C. Lentz, R.E. Chunga, A. Ciarleglio, J.M. Scodes, H. Andrews, P. W. Schofield, Y. Stern, E.D. Huey, K. Bell, G.H. Pelton, Change in odor identification impairment is associated with improvement with cholinesterase inhibitor treatment in mild cognitive impairment, J. Alzheimer's Dis. 60 (4) (2017) 1525–1531.
[16] P. Hasanein, A.K. Mahtaj, Ameliorative effect of rosmarinic acid on scopolamine-induced memory impairment in rats, Neurosci. Lett. 585 (2015) 23–27.
[17] T. Odaira, O. Nakagawasai, K. Takahashi, W. Nemoto, W. Sakuma, J.R. Lin, K. Tan-No, Mechanisms underpinning AMP-activated protein kinase-related effects on behavior and hippocampal neurogenesis in an animal model of depression, Neuropharmacology 150 (2019) 121–133.
[18] O. Nakagawasai, W. Nemoto, H. Onogi, T. Moriya, J.R. Lin, T. Odaira, F. Yaoita, T. Ogawa, K. Ohta, Y. Endo, K. Tan-No, BE360, a new selective estrogen receptor modulator, produces antidepressant and antidementia effects through the enhancement of hippocampal cell proliferation in olfactory bulbectomized mice, Behav. Brain Res. 297 (2016) 315–322.
[19] K. Takahashi, O. Nakagawasai, W. Nemoto, S. Kadota, J. Isono, T. Odaira, W. Sakuma, Y. Arai, T. Tadano, K. Tan-No, Memantine ameliorates depressive-like behaviors by regulating hippocampal cell proliferation and neuroprotection in olfactory bulbectomized mice, Neuropharmacology 137 (2018) 141–155.
[20] K. Takahashi, O. Nakagawasai, W. Nemoto, T. Odaira, W. Sakuma, K. Tan-No, Antidepressant-like effect of aripiprazole via 5-HT1A, D1, and D2 receptors in the prefrontal cortex of olfactory bulbectomized mice, J. Pharmacol. Sci. 137 (3) (2018) 241–247.
[21] S. Hozumi, O. Nakagawasai, K. Tan-No, F. Niijima, F. Yamadera, A. Murata, Y. Arai, H. Yasuhara, T. Tadano, Characteristics of changes in cholinergic function and impairment of learning and memory-related behavior induced by olfactory bulbectomy, Behav. Brain Res. 138 (1) (2003) 9–15.
[22] K. Takahashi, O. Nakagawasai, W. Nemoto, T. Odaira, Y. Arai, T. Hisamitsu, K. Tan-No, Time-dependent role of prefrontal cortex and hippocampus on cognitive improvement by aripiprazole in olfactory bulbectomized mice, Eur. Neuropsychopharmacol. 27 (10) (2017) 1000–1010.
[23] K. Takahashi, O. Nakagawasai, W. Nemoto, T. Odaira, W. Sakuma, H. Onogi, H. Nishijima, R. Furihata, Y. Nemoto, H. Iwasa, K. Tan-No, T. Tadano, Effect of Enterococcus faecalis 2001 on colitis and depressive-like behavior in dextran sulfate sodium-treated mice: involvement of the brain-gut axis, J. Neuroinflamm. 16 (1) (2019) 201.
[24] K. Takahashi, O. Nakagawasai, T. Nakajima, M. Okubo, Y. Nishimura, W. Sakuma, R. Yamagata, W. Nemoto, K. Miyagawa, K. Kurokawa, A. Mochida-Saito, M. Tsuji, H. Takeda, T. Tadano, K. Tan-No, Dopamine D2 receptor supersensitivity in the hypothalamus of olfactory bulbectomized mice, Brain Res. 1746 (2020), 147015.
[25] K. Takahashi, O. Nakagawasai, W. Nemoto, T. Nakajima, Y. Arai, T. Hisamitsu, K. Tan-No, Alterations in behavioral responses to dopamine agonists in olfactory bulbectomized mice: relationship to changes in the striatal dopaminergic system, Psychopharmacology (Berl.) 233 (7) (2016) 1311–1322.
[26] S. Moriguchi, T. Tanaka, H. Tagashira, T. Narahashi, K. Fukunaga, Novel nootropic drug sunifiram improves cognitive deficits via CaM kinase II and protein kinase C activation in olfactory bulbectomized mice, Behav. Brain Res. 242 (2013) 150–157.
[27] J.P. Kelly, A.S. Wrynn, B.E. Leonard, The olfactory bulbectomized rat as a model of depression: an update, Pharmacol. Ther. 74 (3) (1997) 299–316.
[28] S.M. Jancsár, B.E. Leonard, Behavioural and neurochemical interactions between chronic reserpine and chronic antidepressants. A possible model for the detection of atypical antidepressants, Biochem. Pharmacol. 32 (10) (1983) 1569–1571.
[29] C. Song, B.E. Leonard, The olfactory bulbectomised rat as a model of depression, Neurosci. Biobehav. Rev. 29 (4–5) (2005) 627–647.
[30] S. Ahn, M. Choi, H. Kim, E.J. Yang, U. Mahmood, S.I. Kang, H.W. Shin, D.W. Kim, H.S. Kim, Transient anosmia induces depressive-like and anxiolytic-like behavior and reduces amygdalar corticotropin-releasing hormone in a ZnSO4-induced mouse model, Chem. Senses 43 (4) (2018) 213–221.
[31] Y. An, X. Guan, Y. Ni, Y. Zhao, Z. Chen, Y. Chen, J. Zhang, Reversible olfactory dysfunction impaired learning and memory with impaired hippocampal synaptic plasticity and increased corticosterone release in mice, Neurochem. Int. 138 (2020), 104774.
[32] X. Liu, Y. Zhou, D. Yang, S. Li, X. Liu, Z. Wang, Type 3 adenylyl cyclase in the MOE is involved in learning and memory in mice, Behav. Brain Res. 383 (2020), 112533.
[33] K. Takahashi, K. Kurokawa, K. Miyagawa, A. Mochida-Saito, Y. Nemoto, H. Iwasa, O. Nakagawasai, T. Tadano, H. Takeda, M. Tsuji, Antidementia effects of Enterococcus faecalis 2001 are associated with enhancement of hippocampal neurogenesis via the ERK-CREB-BDNF pathway in olfactory bulbectomized mice, Physiol. Behav. 223 (2020), 112997.
[34] G. Paxinos, K.B.J. Franklin, The Mouse Brain in Stereotaxic Coordinates, 2nd ed., Academic Press, San Diego, 2001.
[35] C.A. Duncan-Lewis, R.L. Lukman, R.K. Banks, Effects of zinc gluconate and 2 other divalent cationic compounds on olfactory function in mice, Comp. Med. 61 (4) (2011) 361–365.
[36] K. McBride, B. Slotnick, F.L. Margolis, Does intranasal application of zinc sulfate produce anosmia in the mouse? An olfactometric and anatomical study, Chem. Senses 28 (8) (2003) 659–670.
[37] S.J. Webster, A.D. Bachstetter, P.T. Nelson, F.A. Schmitt, L.J. Van Eldik, Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models, Front. Genet. 5 (2014) 88.
[38] L. Velayudhan, M. Pritchard, J.F. Powell, P. Proitsi, S. Lovestone, Smell identification function as a severity and progression marker in Alzheimer's disease, Int. Psychogeriatr. 25 (7) (2013) 1157–1166.
[39] L. Velayudhan, S. Lovestone, Smell identification test as a treatment response marker in patients with Alzheimer disease receiving donepezil, J. Clin. Psychopharmacol. 29 (4) (2009) 387–390.
[40] P.W. Schofield, H. Ebrahimi, A.L. Jones, G.A. Bateman, S.R. Murray, An olfactory' stress test' may detect preclinical Alzheimer's disease, BMC Neurol. 12 (2012) 24.

[41] N. Mandairon, C.J. Ferretti, C.M. Stack, D.B. Rubin, T.A. Cleland, C. Linster, Cholinergic modulation in the olfactory bulb influences spontaneous olfactory discrimination in adult rats, Eur. J. Neurosci. 24 (11) (2006) 3234–3244.
[42] P.T. Huerta, J.E. Lisman, Bidirectional synaptic plasticity induced by a single burst during cholinergic theta oscillation in CA1 in vitro, Neuron 15 (5) (1995) 1053–1063.
[43] S.V. Adams, J. Winterer, W. Müller, Muscarinic signaling is required for spike-pairing induction of long-term potentiation at rat Schaffer collateral-CA1 synapses, Hippocampus 14 (4) (2004) 413–416.
[44] P. Mohapel, G. Leanza, M. Kokaia, O. Lindvall, Forebrain acetylcholine regulates adult hippocampal neurogenesis and learning, Neurobiol. Aging 26 (6) (2005) 939–946.
[45] X. Liu, Y. Zhou, S. Li, D. Yang, M. Jiao, X. Liu, Z. Wang, Type 3 adenylyl cyclase in the main olfactory epithelium participates in depression-like and anxiety-like behaviours, J. Affect. Disord. 268 (2020) 28–38.
[46] A. Mastrodonato, S.A. Barbati, L. Leone, C. Colussi, K. Gironi, M. Rinaudo, R. Piacentini, C.A. Denny, C. Grassi, Olfactory memory is enhanced in mice exposed to extremely low-frequency electromagnetic fields via Wnt/β-catenin dependent modulation of subventricular zone neurogenesis, Sci. Rep. 8 (1) (2018) 262.
[47] C. Romero-Grimaldi, G. Gheusi, P.M. Lledo, C. Estrada, Chronic inhibition of nitric oxide synthesis enhances both subventricular zone neurogenesis and olfactory learning in adult mice, Eur. J. Neurosci. 24 (9) (2006) 2461–2470.