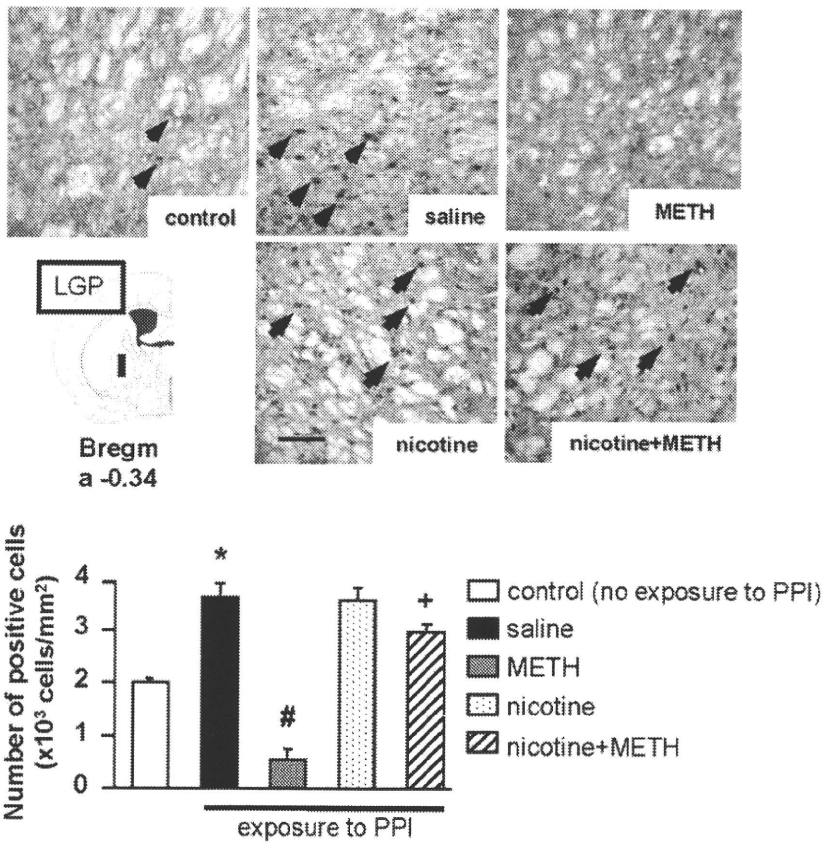

Fig. 4 Effect of nicotine on METH-induced changes in c-Fos expression in LGP after the PPI test. Mice were restricted to the PPI test cage but not subjected to the PPI test (nonexposed control group). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg, sc), nicotine (0.15 mg/kg, sc), or both METH and nicotine. Values are the mean±SE (control, $n=5$; other groups, $n=4$). *$p<0.05$ vs. control group. #$p<0.05$ vs. saline-treated group. +$p<0.05$ vs. METH-treated group

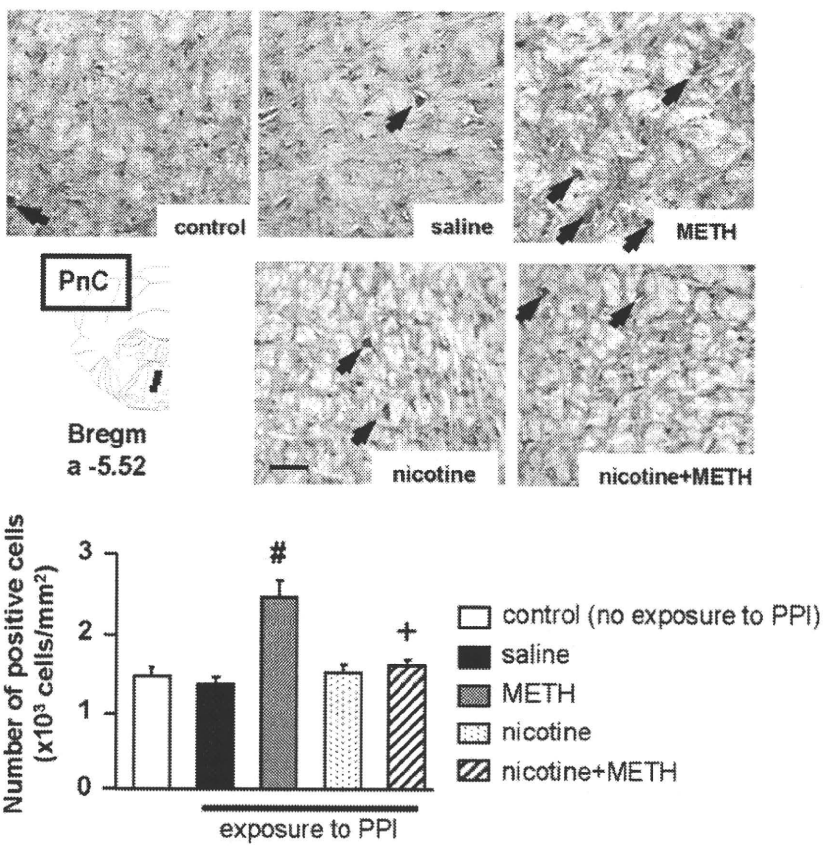

Fig. 5 Effect of nicotine on METH-induced changes in c-Fos expression in the PnC after the PPI test. Mice were restricted to the PPI test cage but not subjected to the PPI test (nonexposed group). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg, sc) nicotine (0.15 mg/kg, sc), or both METH and nicotine. Values are the mean±SE ($n=4$). #$p<0.05$ vs. saline-treated group. +$p<0.05$ vs. METH-treated group. #$p<0.05$ vs. saline-treated group. +$p<0.05$ vs. METH-treated group

The PnC is a critical part of the primary acoustic startle pathway (Fendt et al. 2001). The microinfusion of a nonspecific AchR agonist, carbachol, into the PnC attenuated the startle response and enhanced PPI while the injection of scopolamine reduced PPI (Fendt and Koch 1999). Acetylcholine (Ach) is one of the transmitters of projections from the PPTg to the PnC (Bosch and Schmid 2006) and exerts an inhibitory effect on the tone-evoked activity of acoustically responsive PnC neurons (Koch and Schnitzler 1997; Kungel et al. 1994). Notably, our data showed that nicotine treatment alone had little effect on c-Fos expression in the PnC of saline-treated group after the PPI test (Fig. 5). Although the effect of nicotine on auditory gating appears highly variable, our results suggest that systemic treatment with nicotine at a dose of 0.15–0.5 mg/kg is not directly associated with neuronal activation in the PnC.

Nicotine alters the release of several neurotransmitters, including dopamine, noraderenaline, GABA, glutamate (Dani and De Biasi 2002), and serotonin (Mihailescu et al. 2001, 2002). Interestingly, in the globus pallidus, nicotine can elicit the release of GABA by acting either directly on GABA terminals or indirectly through interaction with a neuronal component such as dopamine, suggesting that nicotine can modulate GABAergic neurons (Kayadjanian et al. 1994). Nicotine activates the neuronal population of the PPTg by directly targeting nicotinic receptors that may be located in noncholinergic neurons including GABAergic and glutamatergic neurons (Lanca et al. 2000). Nicotine stimulation of dorsal raphe nucleus (DRN) alters the activity precisely of the area where the PnC we studied is located, though the release of serotonin (Mihailescu et al. 2002), and nicotine, injected locally into DRN inhibits the activity of pedunculopontine cholinergic neurons through stimulation of DRN serotoninergic neurons, indicating a suppressible effect of nicotine in the PPTg neurons (Mihailescu et al. 2001). Systematic treatment with nicotine may therefore directly or indirectly activate GABAergic neurons in both the LGP and PPTg by altering the release of these neurotransmitters, recovering the function of pallidotegmental GABAergic neurons in METH-treated mice.

Further studies we should carry out are the following: (1) The release of neurotransmitters including Ach, dopamine, GABA, and serotonin in PPTg and PnC after nicotine administration will be examined to make the LGP–PPTg–PnC connection clear; (2) We should look at Fos expression in PPI-regulated and PPI-mediated regions such as superior colliculus (SC), inferior colliculus (IC), and DRN because the PPTg–PnC pathway is modulated by various input–output organization including SC (Kobayashi and Isa 2002; Yeomans et al. 2006), IC, (Yeomans et al. 2006) and DRN (Mihailescu et al. 2001, 2002). PPTg activates SC via nicotinic receptors, which facilitates PPI by activating the fast cascades.

In conclusion, we demonstrated that nicotine ameliorated the impairment of PPI induced by acute treatment with METH through $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptors and reversed the changes in c-Fos expression in both the LGP and PnC to the basal level. In patients suffering from schizophrenia and other psychiatric disorders, PPI is disrupted (Swerdlow et al. 1994; Castellanos et al. 1996). Nicotine receptors may therefore constitute a putative target in the treatment of neuropsychiatric disorders with sensorimotor gating deficits.

Acknowledgments This study was supported in part by a Grant-in-aid for Scientific Research (No.19390062) from the Japan Society for the Promotion of Science and by grants for the 21st century COE program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Smoking Research Foundation, Japan; JSPS and KOSEF under the Japan-Korea Basic Scientific Cooperation Program; the Academic Frontier Project for Private Universities, matching fund subsidy from MEXT, 2007–2011, Research on the Risk of Chemical Substances, Health and Labour Science Research Grants supported by the Ministry of Health, Labour and Welfare, and JST, CREST, and GCOE.

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The Open Behavioral Science Journal, 2010, *4*, 9-18

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Nicotine Ameliorates Emotional and Cognitive Impairments Induced by Neonatal PolyI:C Treatment in Mice

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Abstract: Environmental factors such as maternal and neonatal infection are potentially associated with the pathogenesis of various psychiatric disorders, including schizophrenia. Polyriboinosinic-polyribocytidilic acid (polyI:C) is a synthetic analogue of double-stranded RNA that induces strong innate immune responses. We have recently developed the mouse model of neurodevelopmental psychiatry disorders that exhibits emotional and cognitive impairments in adulthood following neonatal polyI:C treatment. In this study, we examined whether nicotine ameliorates emotional and cognitive impairments in the neonatal polyI:C model because recent studies have indicated the therapeutic benefits of nicotine in schizophrenia. Neonatal ICR mice were repeatedly injected with polyI:C (5 mg/kg, s.c.) for 5 days (postnatal days 2 to 6). At postnatal 10 weeks, emotional functions were analyzed in open field and social interaction tests. Cognitive function was analyzed in novel object recognition and prepulse inhibition (PPI) tests. PolyI:C-treated mice showed an increase in anxiety-like behaviors and impairments in social behaviors, object recognition memory, and PPI, compared with the vehicle-treated control group. Nicotine (0.15 and 0.5 mg/kg, s.c.) dose-dependently improved polyI:C-induced impairments of emotional and cognitive behaviors, but had no effect on PPI deficit. The ameliorating effect of nicotine was antagonized by pretreatment with dihydro-β-erythroidine or methyllycaconitine. These results suggest that nicotine ameliorates emotional and cognitive impairments of the present polyI:C model through nicotinic acetylcholine receptors.

Keywords: Cognition, emotion, nicotine, polyI:C, schizophrenia, nicotinic acetylcholine receptors.

INTRODUCTION

Schizophrenia is a chronic psychiatric disorder characterized by positive and negative symptoms and impaired cognitive function, which affects approximately 1% of the general population [1]. Both genetic factors and environmental insults, including prenatal infection and perinatal complication, are involved in the development of schizophrenia [2]. Recent immunologic, epidemiologic, and neuropsychiatric studies suggest infectious etiologies of several major neuropsychiatric diseases [3]. Infectious organisms that have been implicated in schizophrenia etiology include rubella, influenza, herpes simplex virus, cytomegalovirus, poliovirus, and toxoplasma gondii [4].

Toll-like receptors (TLRs) constitute several families of the pattern-recognition receptors that sense nucleic acids derived from viruses and trigger antiviral innate immune responses [5]. The TLR family consists of more than 13 members in mammals, each detecting different pathogen-associated molecular ligands [5]. In particular, TLR3 recognizes viral double-stranded RNA and host cell mRNA and in turn initiates inflammatory responses [6]. Polyriboinosinic-polyribocytidilic acid (polyI:C) is a synthetic analogue of double-stranded RNA that leads to the pronounced but time-limited production of pro-inflammatory cytokines after it binds to and activates TLR3 [7]. Maternal immune activation by polyI:C exposure in rodents is known to precipitate a wide spectrum of behavioral, cognitive, and pharmacological abnormalities in adult offspring [8-12]. Recently, we have reported that neonatal injection of polyI:C in mice results in schizophrenia-like behavioral alterations, such as emotional and cognitive impairments and dysfunction of glutamatergic neurotransmission in adulthood [13]. We have also proposed a possible interaction of genetic and environmental factors by injecting polyI:C into transgenic mice that express a dominant-negative form of human *disrupted-in-schizophrenia 1*, which is one of the susceptibility genes for schizophrenia [14]. Therefore, polyI:C-treated mice are the useful animal model for schizophrenia that is supported by epidemiological findings.

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1874-2300/10 2010 Bentham Open

Tobacco smoking is frequent among schizophrenia patients [15, 16]. It has been reported that while 25-30% of the population smoke tobacco regularly, 70-90% of chronic schizophrenic patients are tobacco smokers [17, 18]. Nicotine is a potent cholinergic receptor agonist that is inhaled during tobacco smoking. Interestingly, both the expression and function of nicotinic acetylcholine receptors (nAChRs) are down-regulated in the brains of patients with schizophrenia [19, 20]. The enzyme activity of choline acetyltransferase, a biosynthetic enzyme for acetylcholine production is decreased and correlated with poor cognitive function in schizophrenia [21]. It is suggested that the high rate of tobacco smoking among schizophrenia patients represents an attempt to self-medicate, that is, to correct for some disease-associated abnormalities of cholinergic (nicotinic) neurotransmission. Thus, the therapeutic effects of nicotine in schizophrenia have received much interest in recent years.

In this study, to examine whether nicotine ameliorates emotional and cognitive impairments in adult mice that challenged with polyI:C as neonates, neurobehavioral effects of nicotine were analyzed in open field, social interaction, novel object recognition, and prepulse inhibition tests.

MATERIALS AND METHODS

Animals

Timed pregnant ICR mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and maintained under standard specific pathogen-free environmental conditions. Pregnant females were monitored for the parturition date, which was taken as postnatal day (PD) 0. They were housed under a standard 12-h light/dark cycle (lights on at 9:00) at a constant temperature of 23 ± 1°C, with free access to food and water throughout the experiments. We used male mice exclusively to minimize any potential variability due to sex-specific effects in behavioral performance. The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs and Treatment

PolyI:C, (-)-nicotine, dihydro-β-erythroidine [DHβE, an antagonist for α4β2 subunit-containing nAChR (α4β2 nAChR)], and methyllycaconitine [MLA, a selective antagonist for α7 subunit-containing nAChR (α7 nAChR)] were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in saline. All litters were randomly divided into saline and polyI:C-treated groups. From PD 2 to 6, mice were subcutaneously injected daily with either pyrogen-free saline or polyI:C at a dose of 5 mg/kg. Animals were weaned at PD 21, and divided by gender at PD 28. Both groups were derived from multiple litters to preclude possible differences in individual maternal behavior as a mitigating factor in any subsequent long-lasting changes induced in the offspring. Behavioral analyses were started at 10-12 weeks of age in the following order: prepulse inhibition (PPI), open field, novel object recognition test, and social interaction test for studying the effects of nicotine (Figs. **1-4**); open field, novel object recognition test, and social interaction test for studying the effects of nAChR antagonists (Figs. **5-7**). Nicotine (0.15 or 0.5 mg/kg), DHβE (2.0 mg/kg), or MLA (5 mg/kg) was subcutaneously administered 15 min, 25 min, or 25 min, respectively, before behavioral tests. The dose of each drug was in accordance with previous reports [22, 23].

Open Field Test

Mice were placed at the center of an arena and allowed to explore an open field (diameter: 60 cm, height: 35 cm) for 5 min, while their activity was measured automatically using the ethovision automated tracking program (Brainscience Idea Co. Ltd., Osaka, Japan) [24, 25]. The open field was divided into an inner circle (diameter: 40 cm) and an outer area surrounding the inner circle. The movement of mice was measured *via* a camera mounted above the open field. Measurements included distance and time spent in the inner and outer sections.

Social Interaction Test

We used the experimental paradigm described by Tremolizzo *et al.* [26] to measure social behavior (e.g., social interaction, aggression, and escape behavior). PolyI:C-treated or vehicle-treated control mice were individually housed in cages (29 × 18 × 12 cm) for 2 days before the trial. We used 10-12-week-old male ICR mice that had not shown aggressive behavior as intruders. In the first trial (5 min duration), an intruder mouse was introduced into the resident's home cage. The duration of social interaction (close following, inspection, anogenital sniffing, and other social body contacts except aggressive behavior), aggression (attacking/biting and tail rattling), and escape behavior were analyzed. Four trials, with an inter-trial interval of 30 min, were used to analyze social behavior using the same intruder mouse.

Novel Object Recognition Test

A novel object recognition test was carried out as described previously [27]. Mice were individually habituated to an open-box (30 × 30 × 35 high cm) for 3 days. During the training session, two novel objects were placed in the open field and the animals were allowed to explore for 10 min. The time spent exploring each object was recorded. During retention sessions, the animals were placed back into the same box 24 h after the training session, one of the familiar objects used during training was replaced by a novel object, and the mice were allowed to explore the two objects freely for 5 min. The preference index in the retention session, the ratio of the amount of time spent exploring the novel object to the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as the ratio of time spent exploring the object that was replaced by a novel object in the retention session to the total exploration time.

PPI Test

The PPI test was carried out as described previously [28, 29]. After the animals were placed in the chamber (San Diego Instruments, San Diego, California), they were allowed to habituate for 10 min, during which time they

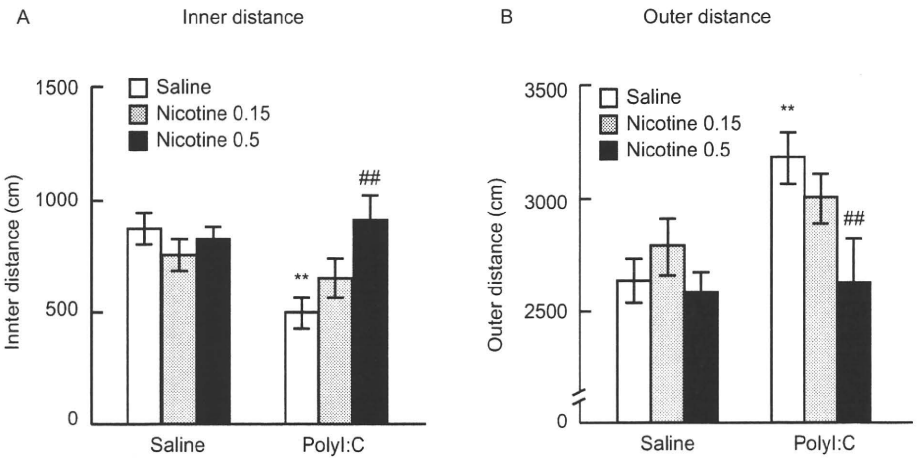

Fig. (1). Effect of nicotine on emotional deficits in open field test in polyI:C-treated mice. Nicotine (0.15 and 0.5 mg/kg, s.c.) was administered 15 min before the behavioral test. Individual mice were allowed to explore the open field freely for 5 min. (A and B) Distance traveled in (**A**) inner and (**B**) outer sectors. Values indicated the mean ± S.E. (n=8-10). **p<0.01 *vs.* saline-treated control group (Fisher's LSD test). ## p<0.01 *vs.* polyI:C-treated control group (Fisher's LSD test).

were subjected to 65 dB background white noise. The animals then received 10 startle trials, 10 no-stimulus trials, and 40 PPI trials. The intertrial interval was between 10 and 20 sec and the total session lasted 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 msec. PPI trials consisted of a prepulse (20 msec burst of white noise at 69, 73, 77, or 81 dB intensity) followed, 100 msec later, by the startle stimulus (120 dB, 40 msec white noise). Each of the four prepulse trials (69, 73, 77, or 81 dB) was carried out 10 times. Sixty different trials were presented pseudo-randomly, ensuring that each trial was carried out 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured for 100 msec after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer, which calculated the maximal response over the 100 msec. Basal startle amplitude was determined as the mean amplitude of the 10 startle trials. PPI was calculated according to the formula: 100 × [1−(PPx/P120)] %, in which PPx was the mean amplitude of the 10 PPI trials (PP69, PP73, PP75, or PP80) and P120 was the basal startle amplitude.

Statistical Analysis

Data are expressed as the mean ± S.E. Statistical significance was determined using analysis of variance (ANOVA) with two-way (Figs. **1-3** and **4B**) or three-way (Fig. **4A**), followed by the Fisher's LSD test when F ratios were significant ($p<0.05$). In Figs. (**5-7**), two-tailed Student's t-test was used between the saline-treated polyI:C and control (saline + saline) groups while two-way ANOVA was performed among polyI:C-treated groups.

RESULTS

Effects of Nicotine on Emotional Deficits in PolyI:C-Treated Mice in Open Field Test

To investigate the effects of nicotine on emotional deficits in polyI:C-treated mice in adulthood, an open field test was carried out at the age of 10-12 weeks, in which the conflict between the drive to explore a new environment and a natural aversion to illuminated open areas was used to examine both anxiety and motor activity [25]. Two-way ANOVA revealed significant effects of polyI:C and nicotine in distance traveled in inner sectors (polyI:C, $F(1,47)=4.24$, $p<0.05$; nicotine, $F(2, 47)=3.45$, $p<0.05$; polyI:C × nicotine interaction, $F(2,47)=4.73$, $p<0.05$, Fig. **1A**) and outer sectors (polyI:C, $F(1,47)=4.17$, $p<0.05$; nicotine, $F(2, 47)=5.10$, $p<0.01$; polyI:C × nicotine interaction, $F(2,47)=2.50$, $p=0.09$, Fig. **1B**) of the open field. The distance traveled in the inner sector of the open field was significantly decreased while the distance traveled in the outer sector was significantly increased in polyI:C-treated mice compared with vehicle-treated control mice ($p<0.01$, Fig. **1A** and **B**). Nicotine dose-dependently and significantly (0.5 mg/kg) increased the distance traveled in the inner sector ($p<0.01$, Fig. **1A**), and decreased the distance traveled in the outer sector in polyI:C-treated mice ($p<0.01$, Fig. **1B**). Likewise, nicotine (0.5 mg/kg) treatment tended to ameliorate the changes in time spent in inner and outer sectors of the open field in polyI:C-treated mice (data not shown). Nicotine itself had no effect on performance in the saline-treated control group (Fig. **1**), suggesting that nicotine has no effect on motor function in mice. These results suggest that nicotine ameliorates polyI:C-induced emotional deficits in adults.

Effects of Nicotine on Deficits of Social Behavior in PolyI:C-Treated Mice

Social interaction in polyI:C-treated mice was investigated at the age of 10-12 weeks. In saline-treated control mice, repeated exposure to an unfamiliar intruder mouse (4 trials) caused a gradual decrease in social interaction time. The polyI:C-treated mice exhibited a marked reduction in the social interaction time in all 4 trials compared with saline-treated control mice (data not shown). Therefore, total social interaction time was evaluated in the following analysis. Two-way ANOVA revealed significant effects of polyI:C and nicotine in social interaction (polyI:C, $F(1,47)=25.27$,

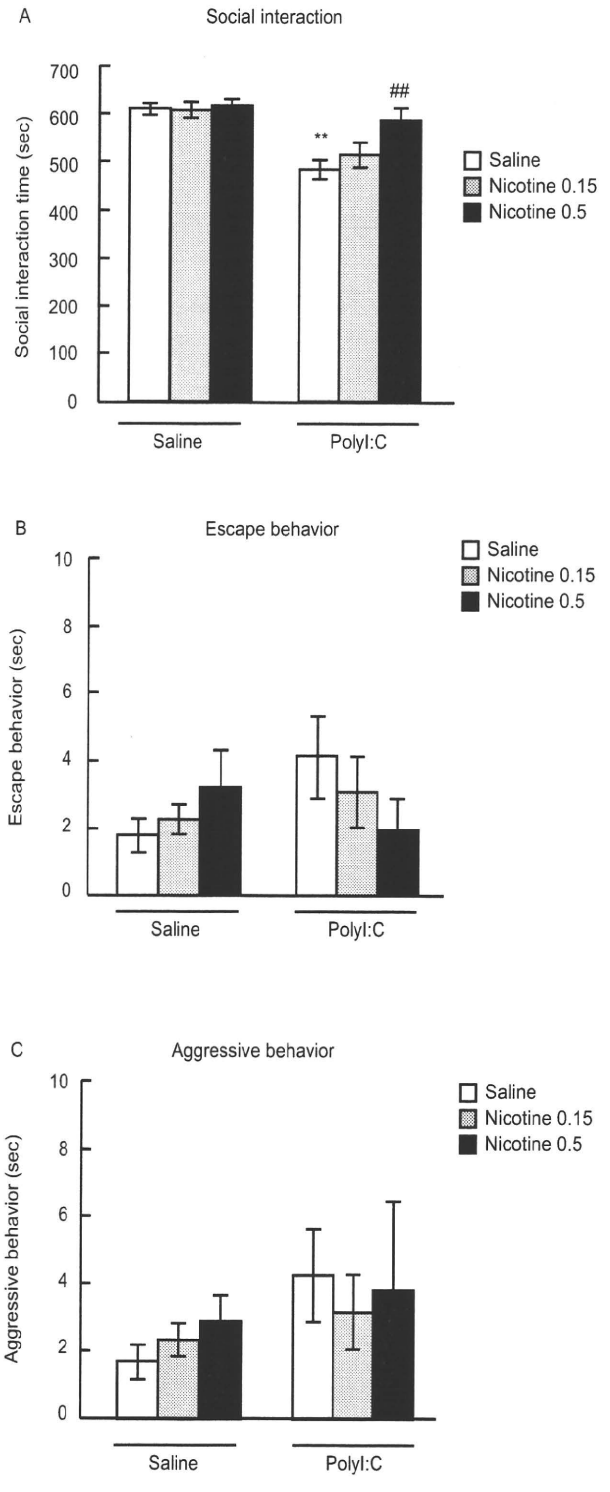

Fig. (2). Effect of nicotine on deficits of social behaviors in social interaction test in polyI:C-treated mice. (**A**) Social interaction, (**B**) escape behavior, and (**C**) aggressive behavior. Nicotine (0.15 and 0.5 mg/kg, s.c.) was administered 15 min before the behavioral test. Values indicate the mean ± S.E. (n=8-10). **p<0.01 *vs.* saline-treated control group (Fisher's LSD test). ## p<0.01 *vs.* polyI:C-treated control group (Fisher's LSD test).

p<0.01; nicotine, F(2,47)=3.82, p<0.05; polyI:C × nicotine interaction, F(2,47)=3.06, p=0.06, Fig. **2A**). These treatments had no effects on escape (polyI:C, F(1,47)=0.68, p=0.41; nicotine, F(2,47)=0.08, p=0.92; polyI:C × nicotine interaction, F(2,47)=1.98, p=0.15, Fig. **2B**) or aggressive behavior (polyI:C, F(1,47)=1.56, p=0.22; nicotine, F(2,47)=0.09, p=0.92; polyI:C × nicotine interaction, F(2,47)=0.26, p=0.77, Fig. **2C**). A single treatment with nicotine (0.5 mg/kg) significantly improved deficits of social interaction in polyI:C-treated mice (p<0.01, Fig. **2A**) without affecting escape (Fig. **2B**) or aggressive behavior (Fig. **2C**). In saline-treated mice, nicotine (0.15 and 0.5 mg/kg) had no effect on social interaction (Fig. **2A**), escape (Fig. **2B**), or aggressive behavior (Fig. **2C**). These results suggest that the ameliorating effect of nicotine on social interaction deficit is not due to locomotor stimulation by the drugs or to changes in escape or aggressive behavior of polyI:C-treated mice.

Effects of Nicotine on Deficits of Object Recognition Memory in PolyI:C-Treated Mice

To examine the effects of nicotine treatment on neonatal polyI:C treatment-induced memory impairment in adults, a novel object recognition test was carried out at the age of 10-12 weeks. During the training session, both polyI:C-treated and saline-treated control mice spent equal amounts of time exploring either one of two objects (two-way ANOVA analysis; polyI:C, F(1,42)=1.48, p=0.23; nicotine F(2,42)=2.62, p=0.09; polyI:C × nicotine interaction, F(2,42) =0.04, p=0.96, Fig. **3B**), and there was no biased exploratory preference in either group (two-way ANOVA analysis; polyI:C, F(1,42)=0.01, p=0.95; nicotine F(2,42)=0.28, p=0.76; polyI:C × nicotine interaction, F(2,42)=0.05, p=0.95, Fig. **3A**), suggesting no differences in motivation and curiosity about novel objects, and motor function between polyI:C-treated and saline-treated control mice. The retention session was carried out 24 h after the training session. Two-way ANOVA indicated that significant effects of polyI:C and nicotine were detected in the level of exploratory preference to the novel object (polyI:C, F(1,42)=51.72, p<0.01; nicotine F(2,42)=11.07, p<0.01; polyI:C × nicotine interaction, F(2,42)=8.70, p<0.01, Fig. **3A**). The level of exploratory preference to the novel object was significantly decreased in polyI:C-treated mice compared with saline-treated control mice (p<0.01, Fig. **3A**). Total exploration time in the retention session did not differ among the groups (two-way ANOVA analysis; polyI:C, F(1,42)=3.70, p=0.06; nicotine F(2,42)=0.35, p=0.71; polyI:C × nicotine interaction, F(2,42)=0.08, p=0.92, Fig. **3B**), indicating that polyI:C-treated mice are unable to discriminate novel and familiar objects. These results suggest that polyI:C-treated mice have impaired recognition memory in adulthood. A single treatment with nicotine (0.5 mg/kg) significantly improved cognitive impairment in polyI:C-treated mice during the retention session (p<0.01, Fig. **3A**) when nicotine was administered 15 min before the training session. Nicotine had no effect on the level of exploratory preference to the novel object in the training session in polyI:C-treated mice (Fig. **3A**). The total exploration time in polyI:C-treated mice was not affected by nicotine in either training or retention session (Fig. **3B**). In saline-treated mice, nicotine had no effect on the level of exploratory preference or total exploration time throughout the experiment (Fig. **3A** and **B**).

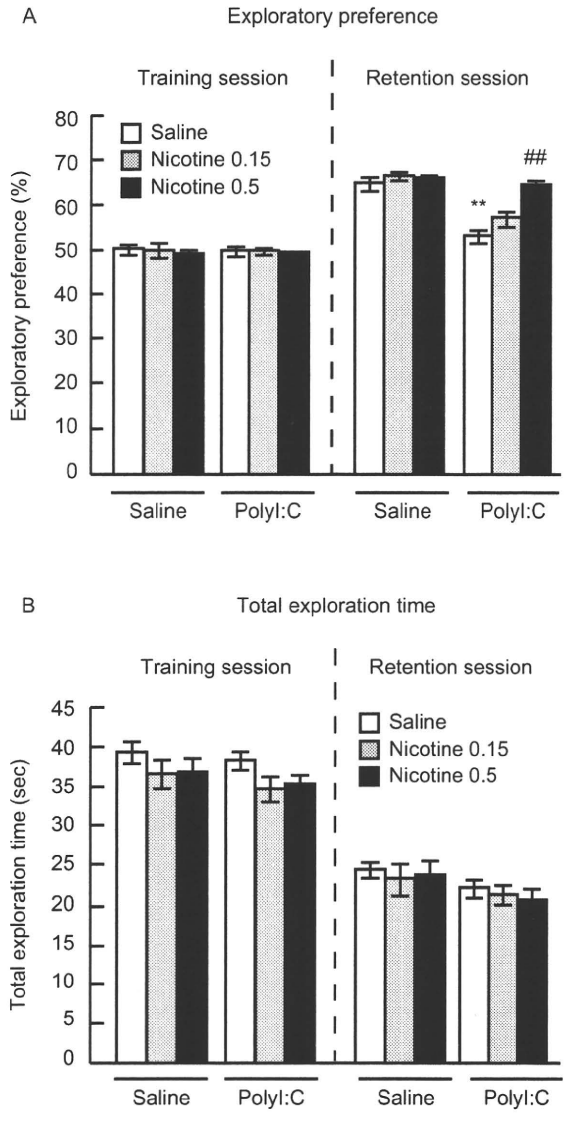

Fig. (3). Effect of nicotine on deficits of object recognition memory in novel object recognition test in polyI:C-treated mice. (**A**) Exploratory preference. (**B**) Total exploration time. Nicotine (0.15 and 0.5 mg/kg, s.c.) was administered 15 min before the training session. The retention session was carried out 24 h after the training session. Values indicate the mean ± S.E. (n=8). **p<0.01 *vs.* saline-treated control group (Fisher's LSD test). ## p<0.01 *vs.* polyI:C-treated control group (Fisher's LSD test).

Effects of Nicotine on PPI Deficits of Startle Response in PolyI:C-Treated Mice

The PPI test was carried out at the age of 10-12 weeks to assess the sensorimotor gating function in polyI:C-treated mice. Three-way ANOVA revealed that significant effects of polyI:C, but no significant effects of nicotine was detected in PPI test (polyI:C, $F(1,42)=72.16$, $p<0.01$; nicotine, $F(2,42)=0.21$, $p=0.81$; polyI:C × nicotine interaction, $F(2,42)=0.14$, $p=0.87$; prepulse, $F(3,126)=55.26$, $p<0.01$; polyI:C × prepulse interaction, $F(3,126)=16.36$, $p<0.01$; nicotine × prepulse interaction, $F(3,126)=0.18$, $p=0.98$; polyI:C × nicotine × prepulse interaction, $F(3,126)=0.67$, $p=0.67$). PolyI:C-treated mice showed a marked impairment of PPI compared with the saline-treated control group at the prepulse intensities (69, 73, 77 and 81 dB) ($p<0.05$ or $p<0.01$, Fig. **4A**). Single treatment with nicotine (0.15 and 0.5 mg/kg) had no effect on PPI deficits (Fig. **4A**) or acoustic startle amplitude in the polyI:C-treated group nor in the saline-treated control group (two-way ANOVA analysis; polyI:C, $F(1,42)=0.06$, $p=0.80$; nicotine, $F(2,42)=0.05$, $p=0.95$; polyI:C × nicotine, $F(2,42)=0.11$, $p=0.89$, Fig. **4B**).

Effects of nAChR Antagonists on Ameliorative Effect of Nicotine Against Emotional and Cognitive Deficits in PolyI:C-Treated Mice

To clarify the subtype of nAChRs involved in the ameliorative effect of nicotine on the emotional and cognitive deficits in polyI:C-treated mice, the mice were pretreated with a selective antagonist for α7 nAChR, MLA, or an antagonist for α4β2 nAChR, DHβE, before nicotine treatment.

In the open field test, two-way ANOVA revealed significant differences in distance traveled in inner sectors (nicotine, $F(1,50)=0.01$, $p=0.93$; nAChR antagonists, $F(2,50)=0.20$, $p=0.82$; nicotine × nAChR antagonists interaction, $F(2,50)=5.91$, $p<0.01$, Fig. **5A**) and outer sectors (nicotine, $F(1,50)=15.96$, $p<0.01$; nAChR antagonists, $F(2,50)=6.83$, $p<0.01$; nicotine × nAChR antagonists interaction, $F(2,50)=0.76$, $p=0.47$, Fig. **5B**). The ameliorating effect of nicotine on anxiety-like behavioral changes in polyI:C-treated mice was completely blocked by pretreatment with either MLA or DHβE ($p<0.05$, Fig. **5A** and **B**). Treatment with MLA or DHβE alone did not affect the change in distance traveled in the inner or outer sector for saline-treated polyI:C mice (Fig. **5A** and **B**). Similar results were observed in the change in time spent in each sector (data not shown).

In the social interaction test, pretreatment with MLA or DHβE significantly attenuated the ameliorating effect of nicotine in polyI:C-treated mice although the same treatment failed to affect saline-treated polyI:C mice (nicotine, $F(1,50)=8.94$, $p<0.01$; nAChR antagonists, $F(2,50)=4.80$, $p<0.05$; nicotine × nAChR antagonists interaction, $F(2,50)=2.63$, $p=0.08$, Fig. **6A**). Furthermore, nAChR antagonists had no effect on escape (nicotine, $F(1,50)=0.05$, $p=0.82$; nAChR antagonists, $F(2,50)=0.01$, $p=0.99$; nicotine × nAChR antagonists interaction, $F(2,50)=0.23$, $p=0.80$, Fig. **6B**) or aggressive behaviors (nicotine, $F(1,50)=0.54$, $p=0.47$; nAChR antagonists, $F(2,50)=0.07$, $p=0.93$; nicotine × nAChR antagonists interaction, $F(2,50)=0.12$, $p=0.89$, Fig. 6C) in either saline-treated or nicotine-treated polyI:C mice.

In the novel object recognition test, MLA and DHβE significantly and completely blocked the ameliorating effect of nicotine on the impairment of object recognition memory in polyI:C-treated mice (nicotine, $F(1,50)=10.85$, $p<0.01$; nAChR antagonists, $F(2,50)=3.66$, $p<0.05$; nicotine × nAChR antagonists interaction, $F(2,50)=5.65$, $p<0.01$, Fig. **7A**). Treatment with MLA or DHβE did not affect the exploratory preference in saline-treated mice or the total

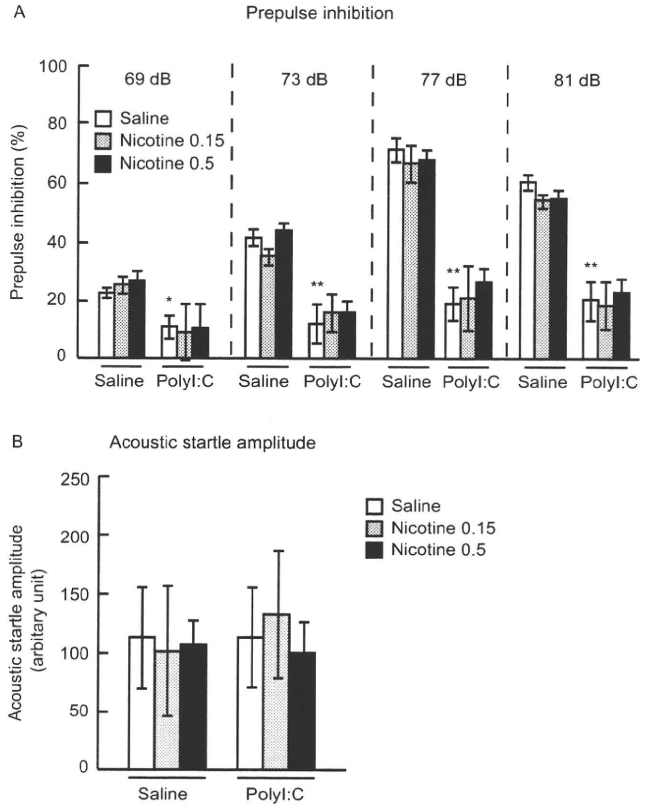

Fig. (4). Effect of nicotine on PPI deficits of startle response in polyI:C-treated mice. (**A**) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (**B**) Acoustic startle amplitude as measured in trials without prepulse. Nicotine (0.15 and 0.5 mg/kg, s.c.) was administered 15 min before the behavioral test. Values indicate the mean ± S.E. (n=8). *p<0.05 and **p<0.01 *vs.* saline-treated control group (Fisher's LSD test).

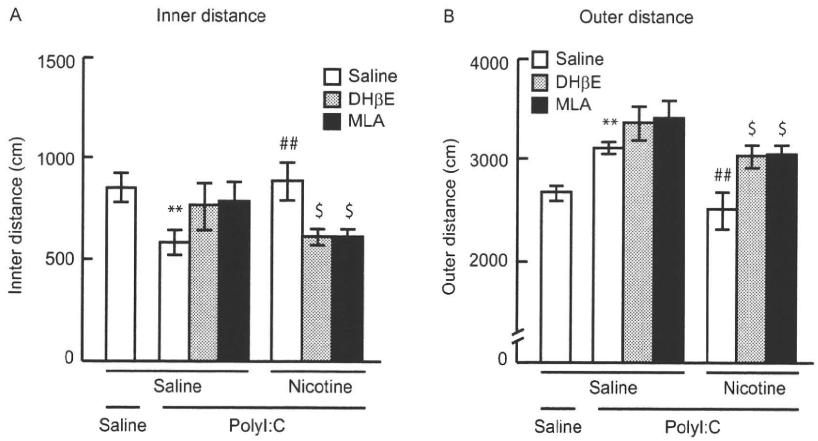

Fig. (5). Effects of nACh receptor antagonists on nicotine-induced amelioration of emotional deficits in polyI:C-treated mice. α4β2 nACh receptor antagonist DHβE (2.0 mg/kg, s.c.) or α7 nACh receptor antagonist MLA (5 mg/kg, s.c.) was administered 25 min before the behavioral test. Nicotine (0.5 mg/kg, s.c.) was administered 15 min before the behavioral test. Individual mice were allowed to explore the open field freely for 5 min. (A and B) Distance traveled in (**A**) inner and (**B**) outer sectors. Values indicate the mean ± S.E. (n=8-16). **p<0.01 *vs.* saline-treated control group (Student's *t*-test). ##p<0.01 *vs.* saline-treated polyI:C group (Fisher's LSD test). $p<0.05 *vs.* nicotine-treated polyI:C group (Fisher's LSD test).

exploration time in either the training or retention sessions in all groups (Fig. **7A** and **B**).

DISCUSSION

It has been reported that the first 2 weeks of postnatal life in the rat and mouse correspond to the second trimester of pregnancy in humans [30], during which time exposure to viral or environmental insult increases the probability of subsequently developing schizophrenia in adolescence. This period is a critical time for neurogenesis in the hippocampus, and for cortical synaptogenesis [31]. According to these findings, we have developed a mouse model of viral infection during the perinatal period by repeatedly injecting

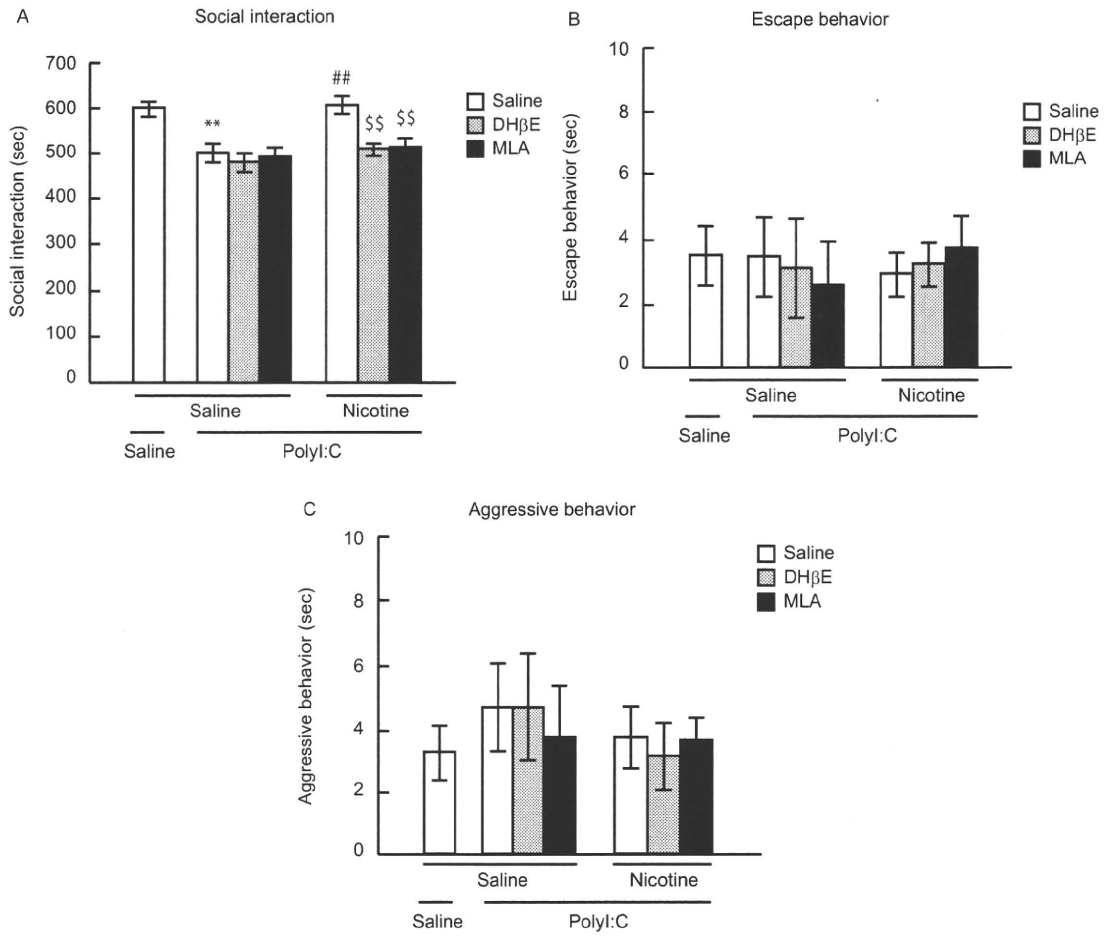

Fig. (6). Effects of nACh receptor antagonists on nicotine-induced amelioration of deficits of social behaviors in polyI:C-treated mice. (**A**) Social interaction, (**B**) escape behavior, and (**C**) aggressive behavior. α4β2 nACh receptor antagonist DHβE (2.0 mg/kg, s.c.) or α7 nACh receptor antagonist MLA (5 mg/kg, s.c.) was administered 25 min before the behavioral test. Nicotine (0.5 mg/kg, s.c.) was administered 15 min before the behavioral test. Values indicate the mean ± S.E. (n=8-16). **p<0.01 *vs.* saline-treated control group (Student's *t*-test). ##p<0.01 *vs.* saline-treated polyI:C group (Fisher's LSD test). $$p<0.01 *vs.* nicotine-treated polyI:C group (Fisher's LSD test).

polyI:C into neonatal ICR mice at postnatal days 2 to 6 [13]. Consistent with our previous study, polyI:C-treated mice showed anxiety-like behavior in the open field test, impaired social behavior in the social interaction test, impaired recognition memory in the novel object recognition test, and sensorimotor gating deficits in the PPI test after puberty. These results suggest that neonatal polyI:C treatment in ICR mice can provide an animal model exhibiting schizophrenia-like behavioral phenotypes after puberty. The abnormal behaviors, except for PPI deficits, in polyI:C-treated mice were ameliorated by the treatment with nicotine in a dose-dependent manner. Thus nicotine may have a therapeutic benefit in ameliorating clinical symptoms in schizophrenia. It is plausible that neonatal polyI:C treatment interferes in the development of cholinergic neurons, leading to abnormal behaviors in adulthood since the early postnatal period is the time for the entry of cholinergic fibers into the cortex [31].

Nicotine failed to improve the PPI deficits in polyI:C-treated mice while smoking transiently normalizes sensory gating deficits in schizophrenia [32]. Studies in rodents have shown that α7 nAChR antagonists induce PPI deficits while α7 nAChR agonists can normalize the auditory gating deficits [33, 34]. The reason for this discrepancy remains unclear. It is unlikely that polyI:C-treated mice lack predictive validity as an animal model of schizophrenia. Because the emotional and cognitive impairments including PPI deficits in polyI:C-treated mice were ameliorated by the treatment of typical and atypical antipsychotics such as haloperidol and clozapine, respectively (data not shown). Alternatively, it is well known that dopamine receptor agonists disrupt PPI of the startle reflex [35]. Therefore, activation of dopaminergic neurons by nicotine [36] may hide the beneficial effect of nicotine on sensorimotor gating in polyI:C-treated mice.

nAChRs are pentameric, ligand-gated ion channels abundant in the central nervous system [37]. Twelve neuronal subunits have been identified, designated α2 to α10 and β2 to β4, which potentially assemble in multiple combinations with a broad range of pharmacological and electrophysiological properties [38]. In particular, the two most prevalent

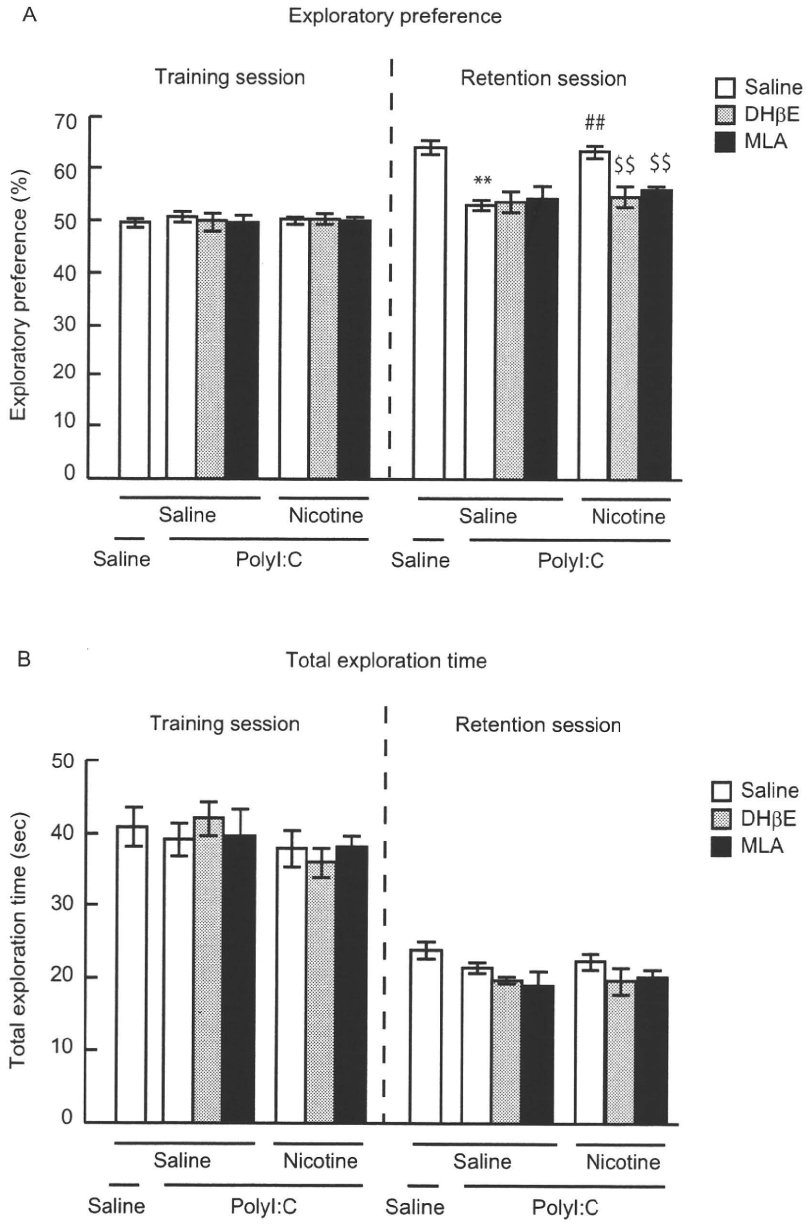

Fig. (7). Effects of nACh receptor antagonists on nicotine-induced amelioration of recognition memory impairment in polyI:C-treated mice. (**A**) Exploratory preference. (**B**) Total exploration time. α4β2 nACh receptor antagonist DHβE (2.0 mg/kg, s.c.) or α7 nACh receptor antagonist MLA (5 mg/kg, s.c.) was administered 25 min before the training session. Nicotine (0.5 mg/kg, s.c.) was administered 15 min before the training session. The retention session was carried out 24 h after the training session. Values indicate the mean ± S.E. (n=8-16). **p<0.01 *vs.* saline-treated control group (Student's *t*-test). ##p<0.01 *vs.* saline-treated polyI:C group (Fisher's LSD test). $$p<0.01 *vs.* nicotine-treated polyI:C group (Fisher's LSD test).

receptors are high-affinity α4β2 nAChR and low-affinity α7 nAChR. High numbers of α4β2 and α7 nAChR binding sites have been observed in several brain regions during the early developmental period [39, 40]. In the present study, we found that both antagonists for α4β2 and α7 nAChRs completely blocked the effect of nicotine on anxiety, and social interaction and memory deficits in polyI:C-treated mice. These results suggest that the ameliorating effects of nicotine on abnormal behaviors in polyI:C-treated mice are mediated by the activation of α4β2 and α7 nAChRs.

It has been reported that the number of α7 nAChRs is reduced in patients with schizophrenia [19, 41]. Functional polymorphisms have been identified in the promoter region of the α7 nAChR gene, which are associated with schizophrenia risk [42, 43]. In addition to α7 nAChR, decreased levels of α4β2 nAChR binding have been found in the hippocampus of patients with schizophrenia [19]. α4β2 nAChR agonists can produce a significant and long-lasting improvement of memory in aged rats and monkeys [44]. Taken together, these findings suggest that α4β2 and α7

nAChRs might be molecular targets for treatment in schizophrenia.

The mechanisms by which nicotine ameliorates the schizophrenia-like behaviors *via* α4β2 and α7 nAChRs remain to be determined. It is known, however, that presynaptic α4β2 and α7 nAChRs stimulate neurotransmitter release, mainly dopamine, glutamate, serotonin, and noradrenaline neurotransmitters [45-49]. Interestingly, our previous study demonstrated that depolarization-evoked glutamate release in the hippocampus of polyI:C-treated mice is significantly lower than the response in saline-treated control mice [13]. Therefore, it is possible that stimulation of α4β2 and α7 nAChRs may trigger glutamate release, contributing to the ameliorating effect of nicotine on emotional and cognitive dysfunction in polyI:C-treated mice. This issue should be resolved in further research.

In conclusion, single treatment with nicotine ameliorated various schizophrenia-like behavioral deficits in polyI:C-treated mice in adulthood although it had no effect on PPI deficits. The antipsychotic effects of nicotine were blocked by α4β2 and α7 nAChR antagonists. These results support the hypothesis that nicotine may have some therapeutic benefits in treating clinical symptoms in schizophrenia.

ACKNOWLEDGEMENTS

We thank Dr. N. Ogiso and the staff at the Division of Experimental Animals, Nagoya University, for their technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research (No. 19390062, 21790067) from the JSPS, Research on the Risk of Chemical Substances, Health and Labor Science Grants supported by the Ministry of Health, Labour and Welfare, the CREST from JST, the MEXT Global-COE Program, Academic Frontier Project for Private Universities, a matching fund subsidy from MEXT, 2007-2011, Regional Joint Research Program supported by grants to Private Universities to Cover Current Expenses from MEXT, and grants from the Smoking Research Foundation.

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Received: December 18, 2009 Revised: March 08, 2010 Accepted: July 05, 2010

Alterations of Emotional and Cognitive Behaviors in Matrix Metalloproteinase-2 and -9-Deficient Mice

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Abstract: Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, and thereby play a crucial role in the remodeling of neural circuits. In the present study, we investigated the role of MMP-2 and MMP-9 in emotional and cognitive function using mice with targeted deletions of the MMP-2 and MMP-9 genes. Emotional behaviors of MMP-9-(-/-) mice but not MMP-2-(-/-) mice were altered, which was manifested in performances in the open-field and elevated plus-arm maze tests. MMP-9-(-/-) mice showed impairments in long-term object recognition memory and conditioned fear memory. MMP-2-(-/-) mice had no deficits in learning and memory. These findings suggest that endogenous MMP-9 play a role in emotional and cognitive behaviors, which may possibly be related to activity-dependent synaptic plasticity and brain development.

Keywords: Matrix metalloproteinase, memory, emotion, behavior.

INTRODUCTION

Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, primarily through the cleavage of extracellular matrix (ECM) proteins and cell-surface components [1]. MMPs constitute a family of enzymes with more than 20 members identified to date, which require Zn^{2+} for their enzymatic activity. Gelatinases (MMP-2 and MMP-9) are capable of cleaving collagen IV and V, laminin, and chondroitin sulfate proteoglycan, which are associated with cell adhesion [1]. MMPs are involved in brain development, because extensive cellular migration and remodeling of the ECM are necessary for neural development [2,3]. Depending on the stage of development, specific and differential expression of MMP and tissue inhibitor of MMP (TIMP) was seen in the cerebellum, which may be related to granular cell migration, arborization of Purkinje cells, and synaptogenesis [4]. Previous studies clearly indicate that a precise knowledge of the relative distribution of the major MMPs is indispensable to delineate their possible role in brain development and plasticity.

The recognition of MMP as a key enzyme in both normal and abnormal nervous system functions represents a rapidly emerging field. Initial studies in this area reported that altered regulation of MMP-2 and MMP-9 was associated with cognitive impairments related to several nervous system disorders. For example, MMP-9 degrades β-amyloid (Aβ) and amyloid plaques [5], and has been implicated specifically in cerebral ischemia [6], kainate-induced neuronal injury [7], and hippocampal long-term potentiation (LTP) and memory [8]. We have also demonstrated that Aβ-induced activation of MMP-9 is related to cognitive impairment induced by Aβ, and that the excessive increase in MMP-9 expression aggravates cognitive impairment [9]. Furthermore, we provided behavioral, neurochemical, and histochemical evidence showing that MMP-2 and MMP-9 are involved in methamphetamine (METH)-induced synaptic plasticity and related behavioral changes by modulating plasmalemmal proteins such as the receptor and transporter [10-12]. Thus, gelatinases are involved in neuronal-activity-dependent synaptic plasticity and cell death in the brain.

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Because of the lack of agents that can selectively inhibit MMP-2 and MMP-9, the physiological roles of endogenous MMP-2 and MMP-9 in brain function such as emotion and memory remain to be determined. To circumvent this problem, we have used mice with targeted deletions of the MMP-2 and MMP-9 genes. We have previously demonstrated that MMP-2 homozygous knock-out [MMP-2-(-/-)] and MMP-9 homozygous knock-out [MMP-9-(-/-)] mice show no difference in locomotor activity compared with their wild-type equivalents [10]. MMP-9 is an inducible protease and expressed in neuronal and grail cells in an activity-dependent manner, while MMP-2 is a constitutive protease. Interestingly, tissue plasminogen activator (tPA) as well as MMP is associated with anxiety-like behavior and memory formation, and some researches demonstrated that tPA in the amygdale promotes stress-induced synaptic plasticity and anxiety-like behavior [13,14]. Thus, it is possible that expression of these proteases can be critical for emotion and memory and that deletion of MMP in mice may exhibit some abnormality in behavioral tests.

In the present study, we evaluated emotional and cognitive behaviors in MMP-2-(-/-) and MMP-9-(-/-) mice. We found that there was some abnormality in performance related to emotionality and long-term memory in MMP-9-(-/-) mice as well as in short-term memory in MMP-2-(-/-) mice. Our findings suggest that the fundamental importance of MMP function in modulating synaptic physiology and plasticity is underscored by behavioral alteration and impairment in emotion and memory displayed in MMP-9-(-/-) mice.

MATERIALS AND METHODOLOGY

Animals

MMP-9-(-/-) mice and equivalent wild-type (FVB/N) mice (10 weeks old) obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at the beginning of the experiments were used. We also used MMP-2-(-/-) mice and equivalent wild-type (C57BL/6J) mice (10–12 weeks old) [15]. These mutant and wild-type mice used in the present study were littermates, and only male mice were used in behavioral test. The animals were housed in plastic cages and kept in a regulated environment (23 ± 1°C, 50 ± 5% humidity) with a 12 h light-dark cycle (lights on at 9:00 am). Food and tap water were available ad libitum.

All experiments were performed in accordance with the Guidelines for Animal Experiments of the Kanazawa University and Nagoya University Graduate School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Open-Field Test

The open field test was carried out as described previously [16], with minor modifications. The open-field used in the study consisted of a circular area with gray walls (60 cm diameter, 60 cm high) and was set in a dark, sound-attenuated room. The floor of the field was divided into one start circle (20 cm diameter) and 18 identical areas so that the animal's ambulation could be measured. The field was divided into inner (40 cm diameter) and outer sectors. A light (100 W) was positioned 100 cm above the center of the floor of the apparatus. Each mouse (n=10 for MMP-9-(-/-) and their wild-type equivalents; n=5 for MMP-2(-/-) and their wild-type equivalents) was placed in the center of the open field. The mice were allowed to freely explore the environment for 10 min. During this time, the ambulation of the mice was measured by counting the number of times that the animals crossed from one area to another. We measured the time spent visiting the inner sector and the number of entries into the inner sector (inner sector visits). The numbers of rearing, climbing, and grooming events were also recorded.

Elevated Plus-Arm Maze Test

The elevated plus-arm maze consisted of two open (30x5 cm) and two closed arms (30x5x25 cm) emanating from a common central platform (5x5 cm) to form a plus shape. The entire apparatus was elevated to a height of 40 cm above floor level. Testing commenced by placing a mouse (n=10 for MMP-9-(-/-) and their wild-type equivalents; n=5 for MMP-2(-/-) and their wild-type equivalents) on the central platform of the maze facing an open arm, and a standard 5-min test duration was employed. Conventional parameters consisted of the numbers of open and closed arm entries and the time spent in the open arms.

Novel-Object Recognition Test (NORT)

The NORT was carried out as described previously [18,19]. The experimental apparatus consisted of a Plexiglas open-field box (30x30x35 cm), with a sawdust-covered floor. The apparatus was located in a sound-attenuated room and was illuminated with a 20 W bulb.

In a standard procedure, the NORT consisted of three sessions: habituation, training, and retention. Each mouse (n=10 for MMP-9-(-/-) and their wild-type equivalents; n=8-10 for MMP-2(-/-) and their wild-type equivalents) was individually habituated to the box, with 10 min of exploration in the absence of objects for 3 consecutive days (habituation session, days 1–3). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore the box for 10 min (day 4). The objects were constructed from a golf ball, a wooden column, and a wall socket, which were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was facing the object and/or it was touching or sniffing the object. The time spent exploring each object was recorded, and the mice were immediately returned to their home cages after training. The animals were placed back into the same box once, either 1 or 24 hr after the training session, to assess short-term and long-term memory, respectively (retention session). During the retention sessions, one of the familiar objects used during training had been replaced with a novel object. The animals were then allowed to explore freely for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index in the retention session, a ratio of the amount

of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as the ratio of the time spent exploring the object that was replaced by the novel object in the retention session over the total exploring time.

Cued and Contextual Fear Conditioning Tests

Cued and contextual fear conditioning tests were performed in accordance with previous reports [17], with minor modifications. Freezing behavior, which shows mice's head, arms and legs not moving, was measured by stop-watch. For measuring basal levels of freezing response (preconditioning phase), mice (n=8-11) were individually placed in a neutral cage (a block Plexiglas box with abundant wood tips, 30x30x35 cm) for 1 min, then in a conditioning cage (a transparent Plexiglas box, 30x30x35 high cm) for 2 min. For training (conditioning phase), mice were placed in the conditioning cage, then a 15 s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea Co., Ltd.). This procedure was repeated 4 [MMP-2-(-/-)] or 8 [MMP-9-(-/-)] times with 15 s intervals. Cued and contextual tests were carried out 1 day after fear conditioning. For the contextual test, mice were placed in the conditioning cage and the freezing response was measured for 2 min in the absence of the conditioned stimulus. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus.

Statistical Analysis

All data were expressed as the mean ± SE. Statistical significance was determined using Student's t-test for two-group comparisons, or a one-way analysis of variance (ANOVA), followed by the Fisher' LSD test for multigroup comparisons. P values less than 0.05 were taken to indicate statistically significant differences.

RESULTS

Performance in Open-Field and Elevated Plus-Arm Maze Tests by MMP-9-(-/-) and MMP-2-(-/-) Mice

There was a clear strain difference of performance in open-filed and elevated plus-arm maze test between FVB/N mice and C57BL/6J mice (Fig. **1** and Fig. **2**). It was mani-

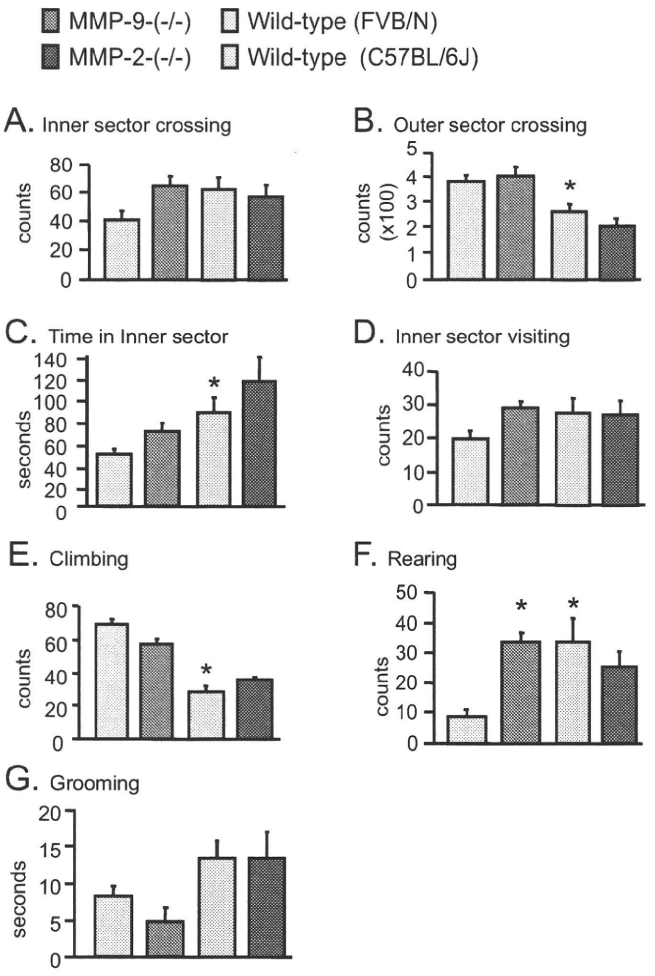

Fig. (1). Performance in the open-field test by MMP-2-(-/-) mice and MMP-9-(-/-) mice. In the open-field test, each mouse was allowed to freely explore the environment for 10 min. Values are the mean±S.E. (n=10 for MMP-9-(-/-) and their wild-type equivalents, n=5 for MMP-2(-/-) and their wild-type equivalents). *p<0.05 *vs.* wild-type (FVB/N) mice.

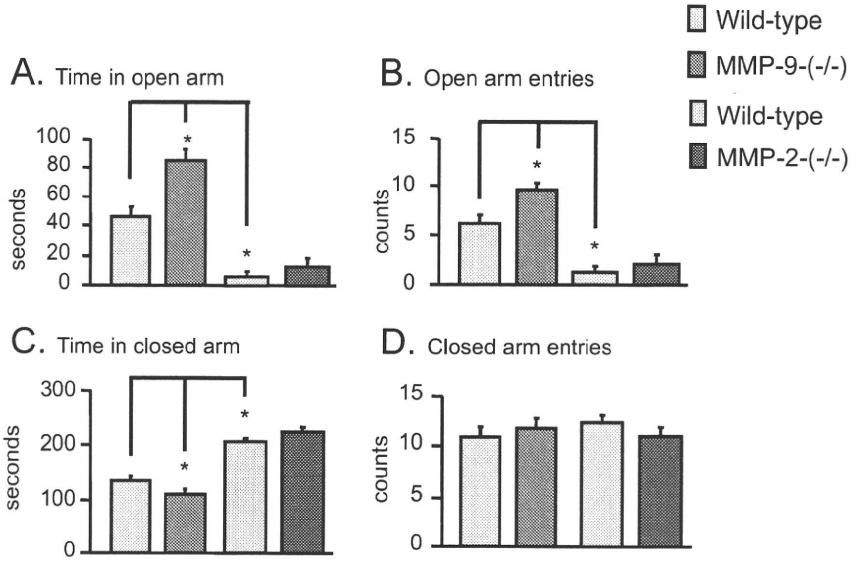

Fig. (2). Performance in the elevated plus-arm maze test by MMP-2-(-/-) mice and MMP-9-(-/-) mice. In the elevated plus-arm maze test, each mouse was allowed to freely explore the maze for 5 min. Values are the mean±S.E. (n=10 for MMP-9-(-/-) and their wild-type equivalents, n=5 for MMP-2(-/-) and their wild-type equivalents). *p<0.05 *vs.* wild-type (FVB/N) mice.

fested by the changes in outer sector crossing (Fig. **1B**, $F(3,26)=7.67$, $p<0.05$), time in inner sector (Fig.**1C**, $F(3,26)=6.34$, $p<0.05$), climbing (Fig.**1E**, $F(3,26)=9.57$, $p<0.05$), rearing (Fig.**1F**, $F(3,26)=6.82$, $p<0.05$) in the open-field test as well as the changes in time in open arm (Fig. **2A**, $F(3,26)=27.7$, $p<0.05$), open arm entries (Fig. **2B**, $F(3,26)=19.7$, $p<0.05$), and time in closed arm (Fig. **2C**, $F(3,26)=33.2$, $p<0.05$) in the elevated plus-arm maze test. Because it is obvious that we cannot compare the effects of targeted deletions of MMP-2 and MMP-9 genes on emotional behaviors, the comparison was made with the same strain.

When MMP-9-(-/-) mice were exposed to a novel environment under mild stressful conditions in the open-field test, they showed a significantly increased number of rearing events compared with wild-type mice (Fig.**1F**, $p<0.05$). The mutant mice showed a tendency to explore the inner sector of open-filed more than did wild-type mice, as indicated by an increased inner sector crossing (Fig. **1A**), time in the inner sector (Fig. **1C**) and inner sector visiting (Fig. **1D**). But these alterations were not statistically significant. There was no difference in any behavioral events between wild-type and MMP-2-(-/-) mice in the open-field test (Fig. **1**).

To further evaluate emotional change, MMP-9-(-/-) and MMP-2-(-/-) mice were subjected to the elevated plus-arm maze test. The time spent in open arms and open arm entries by MMP-9-(-/-) mice was significantly longer than those by wild-type mice while that in closed arms was shorter than that by wild-type mice (Fig.**2A**, $F(3,26)=27.7$, $p<0.05$; **2B**, $F(3,26)=19.7$, $p<0.05$; **2C**, $F(3,26)=33.2$, $p<0.05$). In contrast, there was no difference in performance in the elevated plus-maze test between wild-type and MMP-2-(-/-) mice (Fig. **2**).

Alternation Behavior in Y-Maze task in MMP-9-(-/-) and MMP-2-(-/-) Mice

We evaluated short-term working memory in MMP-9-(-/-) and MMP-2-(-/-) mice in a Y-maze test. One-way ANOVA revealed that there was a significant difference in the numbers of arm entries ($F(3,28)=5.78$, $p<0.05$), but not in spontaneous alternation behavior (%) ($F(3,28)=2.51$, $p>0.05$) among 4 groups of animals. The post-hoc analysis of arm entries indicated that there were no differences between FVB/N mice and C57BL/6J mice or between mutant mice and the respective wild-type mice (data not shown).

Recognition Memory in MMP-9-(-/-) and MMP-2-(-/-) Mice

We used a novel-object recognition test to assess short-term and long-term recognition memory [18,19]. In the 1-hr retention session, MMP-9-(-/-) and MMP-2-(-/-) mice exhibited levels of exploratory preference and exploration time for the novel objects similar to those of their respective wild-type mice, suggesting no impairment of short-term memory (data not shown).

When retention performance was tested 24 hr after the training session, one-way ANOVA indicated a significant difference in the level of exploratory preference for the novel objects among 4 groups of animals (Fig. **3**, $F(3,34)=7.19$, $p<0.05$). Post-hoc analysis indicated that the exploratory preference for the novel objects in MMP-9-(-/-) mice was significantly decreased compared with that in the wild-type mice ($p<0.05$). There were apparent differences in total exploration time during the training (Fig. **3A**, $F(3,34)=18.1$, $p<0.05$) and retention sessions (Fig. **3A**, $F(3,34)=35.9$, $p<0.05$) between FVB/N mice and C57BL/6J mice, but no difference was observed between mutant mice and the respective wild-type mice. There was no difference in exploratory preference and exploration time in training and retention session between MMP-2-(-/-) and wild-type mice (Fig. **3B**). These results suggest that MMP-9-(-/-) mice but not MMP-2-(-/-) mice have the deficit in long-term memory retention but that the memory acquisition (learning) and short-term memory of MMP-9-(-/-) mice are not impaired.

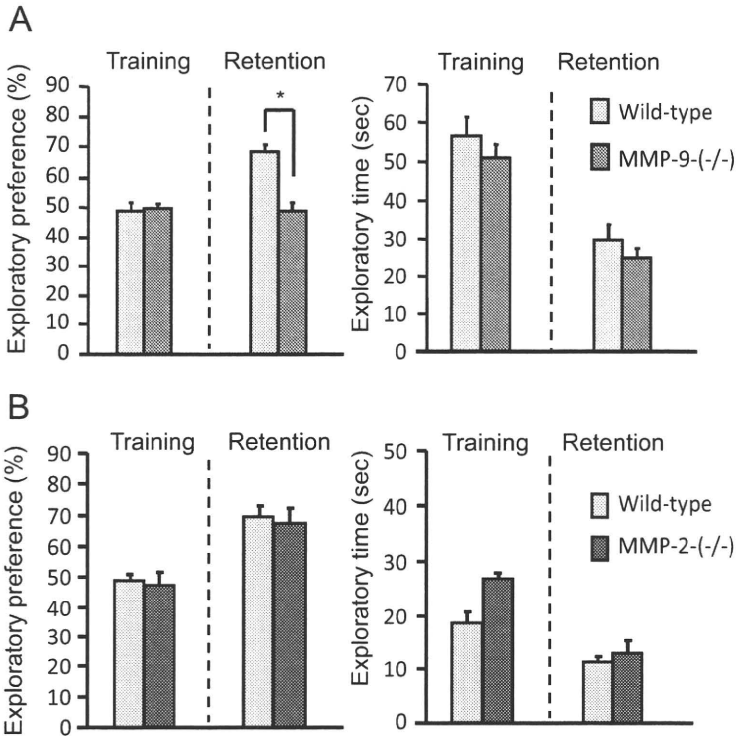

Fig. (3). Performance in the long-term NORT by MMP-9-(-/-) (**A**) and MMP-2-(-/-) (**B**) mice. The retention session was carried out 24 hr after the training. Values are the mean±S.E. (n=10 for A, n=8-10 for B). *p<0.05 *vs.* wild-type mice.

Conditioned Fear Memory in MMP-9-(-/-) and MMP-2-(-/-) Mice

We evaluated associative learning in a conditioned fear learning test. In the preconditioning phase, all groups of mice hardly showed any freezing response. Because different protocols for the training were used in FVB/N mice and C57BL/6J mice, statistical comparison was made between the mutant mice and their respective wild-type mice.

MMP-9-(-/-) mice exhibited less freezing response than their wild-type equivalents in the cued (Fig. **4A**, $p<0.05$) and contextual test (Fig. **4B**, $p<0.05$), indicating an impairment of associative learning. There was no difference in the cued and contextual freezing responses between MMP-2-(-/-) and wild-type mice (Fig. **4**). There was an obvious difference in the minimal current required to elicit flinching/running, jumping, or vocalization between FVB/N mice and C57BL/6J mice, but no alterations were found between mutants and their respective wild-type equivalents (MMP-9-(-/-): 0.31± 0.02 mA, wild-type: 0.33±0.01 mA; MMP-2-(-/-): 0.16±0.03 mA, wild-type: 0.19±0.02 mA).

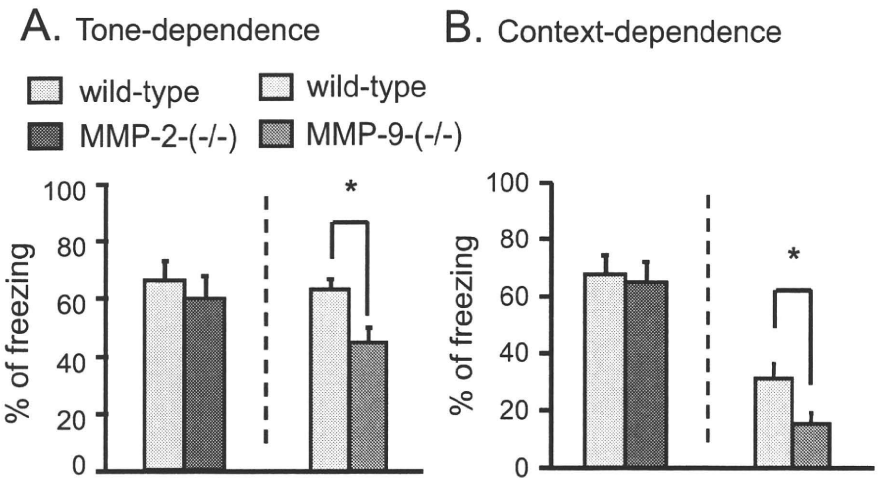

Fig. (4). Performance in the conditioned fear learning test by MMP-9-(-/-) and MMP-2-(-/-) mice. The retention session was carried out 24 hr after the training. Cue-dependent (**A**) and context-dependent (**B**) freezing times were measured. Values are the mean±S.E. (n=8-11). *p<0.05 *vs.* wild-type mice.

DISCUSSION

The present study investigated the role of MMP-2 and MMP-9 in emotional and cognitive function by assessing the performance of mice with targeted deletions of the MMP-2 and MMP-9 genes in various behavioral tasks. MMP-9-(-/-) mice but not MMP-2(-/-) mice showed a behavioral response indicating reduced anxiety upon exposure to mild stressful situations. The emotional changes were manifested by increased numbers of rearing in the open-field test, and increased time spent in the open arms and more open arms entries in the elevated plus-arm maze test. Accordingly, it is plausible that endogenous MMP-9 may be involved in the modulation of emotional behavior. However, a previous report showed that MMP inhibition disrupted reconsolidation of the fear memory, which is related to emotionality in a reactivation-dependent manner, and that the reduced freezing behavior was not due to a decrease in general anxiety levels, since FN-439, a broad-spectrum metalloproteinase inhibitor, had no effect on the time spent in open arms or on the numbers of open arm entries in an elevated plus-arm maze task, suggesting that MMP may be involved in fear memory, but not anxiety-like fear [20]. Therefore, further experiments are necessary with various anxiety tests, since an anxiogenic behavioral response could be tied to specific stimuli, influenced by motor factors, and situation-specific. Additionally, it is well known that phenotypic changes in behavior of mutant mice are dependent, at least in part, on the genetic background, C57BL/6J and FVB/N. Thus, we have to interpret the results in the present study with caution.

MMP-9-(-/-) mice showed an impairment of long-term object recognition and fear memory, while spontaneous alternation behavior in the Y-maze test and short-term memory were intact. These results imply that MMP-9 plays a role in the formation of long-term but not short-term memory. In agreement with our results, the infusion of an MMP inhibitor into the dorsal hippocampus was found to disrupt acquisition of spatial memory in Morris's water maze [21]. Hippocampal MMP-9 expression is increased transiently during water maze acquisition, and inhibition of MMP activity with MMP-9 antisense oligonucleotides and MMP inhibitor altered LTP and prevented acquisition of spatial memory in Morris's water maze [22]. Furthermore, Nagy *et al.* [8] showed that MMP-9 null-mutant mice display a significant deficit in long-term hippocampus-dependent memory for context but not cued conditioning compared with their wild-type equivalents. These results indicate that changes in MMP function are critical in synaptic plasticity and hippocampal-dependent memory and that compromising the ability of the dorsal hippocampus to reconfigure ECM molecules by inhibiting MMP activity interferes with appropriate spatial memory acquisition. In contrast to the report by Nagy *et al.*, our findings showed that hippocampus-independent cued fear memory was also disrupted in MMP-9-(-/-) mice compared with that in wild-type mice, indicating that the functioning of the corticolimbic system including the amygdala is disrupted in MMP-9-(-/-) mice. The discrepancy between our findings and those of previous studies may reflect differences in the methodology of the fear memory test and the types of mutant mice used.

Activity-dependent changes in the organization of ECM are considered to alter synaptic architecture and physiology in a way that changes the efficiency of synaptic transmission. Recent study has identified that MMP-9 as a physiological regulator of N-methyl-$_D$-aspartate (NMDA) receptor-dependent synaptic plasticity and memory. This mechanism of MMP-9 action on NMDA receptors is not mediated by change in overall ECM structure nor by direct cleavage of NMDA receptor subunits, but rather through an integrin beta1-dependent pathway [23]. The NMDA receptor is an important mediator of synaptic plasticity and plays a central role in the neurobiological mechanisms of emotionality, as well as learning and memory [24]. In fact, we have demonstrated that MMP-9 plays a role in METH-induced behavioral sensitization and reward as well as dopamine release through modulation of the function of dopamine receptors and transporters [10,11].

MMP, by focalized and controlled proteolysis, may be crucial in determining the hierarchy of processes involved in brain development and plasticity. In the cerebellum, there is differential and spatiotemporal expression of MMP-2 and MMP-9 during its development [4]. These regional and cellular expression patterns are evidence for tightly regulated proteolysis-mediated alteration of ECM components, which might be related to the migration of granular precursors and Purkinje cell arborization. The spatiotemporal regulation of MMPs may be crucial in brain maturation and plasticity. Accordingly, we cannot exclude the possibility that neuroadaptation and compensatory mechanisms for the targeted deletions of the MMP-2 and MMP-9 genes may contribute to the altered emotional and cognitive behaviors in the mutant mice, although histochemical examinations revealed no major defects in brain structure.

In conclusion, the present study demonstrates that mice with targeted deletions of the MMP-9 gene show deficits in emotional and cognitive behaviors while the deletion of the MMP-2 gene has no effect on these behaviors. These results suggest that endogenous MMPs, especially MMP-9, have a role in the regulation of emotion and memory.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-aid for Scientific Research (Nos. 19390062 and 21790068) from the Japan Society for the Promotion of Science, a grant from the Japan Epilepsy Research Foundation, the Suzuken Memorial Foundation, the Kanzawa Medical Research Foundation and by grants for the global COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Academic Frontier Project for Private Universities matching fund subsidy from MEXT, 2007-2011, Research on the Risk of Chemical Substances, Health and Labour Science Research Grants supported by the Ministry of Health, Labour and Welfare, the Regional Joint Research Program supported by grants to Private Universities to Cover Current Expenses from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and JST, CREST.

ABBREVIATIONS

Aβ = β-amyloid

ECM = Extracellular matrix

LTP = Long-term potentiation

METH = Methamphetamine

MMPs = Matrix metalloproteinases

NORT = Novel-object recognition test

NMDA = *N*-methyl-$_D$-aspartate

TIMP = Tissue inhibitor of MMP

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Received: December 18, 2009 Revised: March 08, 2010 Accepted: July 05, 2010