Moreover, we recently reported that neonatal injection of polyI:C in mice results in schizophrenia-like behavioral alterations in adulthood [11]. In the publication, we discussed the rationale in choosing the timing of polyI:C injection during the mouse neonatal stage that corresponds to the human second trimester [11]. Accordingly, neonatal DN-DISC1 mice were repeatedly injected with polyI:C for 5 days from postnatal day 2 to 6, which correspond to post conception day 128–158 for cortical events and 93–115 for limbic events of brain development in humans (http://translatingtime.net; see also [7]).

In the present study, we demonstrate that combined effect of neonatal polyI:C treatment and DN-DISC1 affects some behavioral and anatomical phenotypes in adulthood. Of note, as far as we are aware, this is the first experimental demonstration that "neonatal" interaction of major genetic and environmental susceptibility factors for schizophrenia results in the dramatic change in the parvalbumin-positive interneurons in the medial prefrontal cortex (mPFC), one of the best hallmarks for schizophrenia [17,20].

2. Materials and methods

2.1. Animals

Transgenic mice expressing a dominant-negative mutant DISC1 under the expression control of CaMKII promoter (DN-DISC1; line 10) [10] were used in this study. Littermates (both males and females) generated by cross breeding of wild-type (WT, C57BL/6N) female and DN-DISC1 male mice were used for experiments. They were housed under a standard 12-h light/dark cycle (light phase 9:00–21:00) at a constant temperature of 23 ± 1 °C, with free access to food and water throughout the experiments.

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.

2.2. Treatment

All litters were randomly divided into two groups: vehicle and polyI:C-treated mice. Mice were treated by subcutaneous injection with either pyrogen-free saline or 5 mg/kg polyI:C (Sigma–Aldrich, St. Louis, MO) daily between postnatal days 2 and 6. Animals were weaned at postnatal day 21, and divided along gender lines at postnatal day 28. Four groups [e.g., vehicle-treated WT (vehicle/WT), polyI:C-treated WT (polyI:C/WT), vehicle-treated DN-DISC1 (vehicle/DN-DISC1) and polyI:C-treated DN-DISC1 (polyI:C/DN-DISC1)] were derived from multiple (at least 3) litters to preclude possible differences in individual maternal behaviors as a mitigating factor in any subsequent long-lasting changes induced in the offspring. Behavioral analyses were started at 8 weeks old, and carried out in the following orders: Y-maze test, novel object recognition test, prepulse inhibition test, fear conditioning test, social interaction test and MK-801-induced hyperactivity.

2.3. Behavioral assays

2.3.1. Y-maze test

Y-maze test was carried out as described previously [10]. Each arm is 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The arms converge in an equilateral triangular central area that is 4 cm at its longest axis. Each mouse is placed individually at the center of the apparatus and allowed to move freely through the maze during an 8-min session. The series of arm entries is recorded visually. Alternation is defined as successive entries into the three arms, on overlapping triplet sets. The percent alternation is calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus 2) multiplied by 100. Spontaneous alternation (%) defined as successive entries into the three arms on overlapping triplet sets is associated with the capacity of short-term memory.

2.3.2. Novel object recognition test

A novel object recognition test was carried out as described previously [11]. Mice were individually habituated to an open-box (30 cm × 30 cm × 35 high cm) for 3 days. During the training session, two novel objects were placed into 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 1 h after the training session, in which one of the familiar objects used during training was replaced by a novel object, and allowed to explore freely for 5 min. 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 a ratio of the time spent exploring the object that was replaced by the novel object in the retention session, over the total exploring time.

2.3.3. Prepulse inhibition test

The prepulse inhibition (PPI) test was carried out as described previously [11]. After the animals were placed in the chamber under moderately bright light conditions (180 lux) (San Diego Instruments, San Diego, CA), they were allowed to habituate for 10 min, during which 65 dB background white noise was present. The animals then received 10 startle trials, 10 no-stimulus trials and 40 PPI trials. The intertrial interval was between 10 and 20 s and the total session lasted 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 ms. PPI trials consisted of a prepulse (20 ms burst of white noise at 69, 73, 77 or 81 dB intensity) followed, 100 ms later, by the startle stimulus (120 dB, 40 ms white noise). Each of the four prepulse trials (69, 73, 77 or 81 dB) was presented 10 times. Sixty different trials were presented pseudo-randomly, ensuring that each trial was presented 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured for 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 10 startle trials. PPI was calculated according to the formula: $100 \times [1 - (PPx/P120)]$ %, in which PPx was the mean of the 10 PPI trials (PP69, PP73, PP75 or PP80) and P120 was the basal startle amplitude.

2.3.4. Fear conditioning test

To examine contextual memory in polyI:C/DN-DISC1 mice, we used context-dependent conditioned fear test according to previous report [1]. In the conditioning phase, each mouse is placed in the training cage (30 cm × 30 cm × 40 cm) equipped with a metal floor, and a 15-s tone (85 dB) is delivered (conditioned stimulus). During the last 5 s of the tone stimulus, a foot shock of 0.8 mA is delivered as an unconditioned stimulus through a shock generator. This procedure is repeated four times with 15 s intervals. Twenty-four hours after the conditioning, context-dependent test was carried out. For context-dependent test, mouse is placed in the training cage, and the freezing response is measured for 2 min in the absence of the conditioned stimulus. Two hours after context-dependent test, tone-dependent test was carried out. For tone-dependent 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 using mice which had been subjected to context-dependent test.

2.3.5. Social interaction test

We used the experimental paradigm described by Ibi et al. [11] to measure social behavior (e.g., social interaction, aggression and escape behavior). Vehicle/WT and polyI:C/DN-DISC1 mice were individually housed in a home cage (29 cm × 18 cm × 12 cm) for 2 days before the trial. We used 10–15-week-old male C57BL mice as intruders which had not shown aggressive behavior. 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.

2.4. MK-801-induced hyperactivity

To investigate the possible changes in sensitivity of glutamate N-methyl D-aspartate (NMDA) receptor in polyI:C/DN-DISC1 mice, locomotor activity induced by MK-801 (Sigma–Aldrich) was measured as described previously [11]. Each mouse was placed in a standard transparent rectangular rodent cage (25 cm × 30 cm × 18 cm) and allowed a 120-min habituation period before MK-801 (0.3 mg/kg, i.p.) treatment. Locomotor activity was then measured for 180 min immediately after MK-801 treatment, using an infrared sensor (NS-AS01; Neuroscience, Tokyo, Japan) placed over the cage.

2.5. Histological analyses

In histological analysis, 8-week-old mice that had not previously subjected to behavioral analysis were deeply anesthetized with diethyl ether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Their brains were removed, post-fixed in the same fixative, and then cryoprotected. Thirty micrometer-thick coronal brain sections were cut on a cryostat and mounted on slides. The section used for the analysis of the mPFC was collected between stereotaxic coordinates bregma 1.78 and 1.54 according to the brain atlas [21] and those in the hippocampus was collected according to Ibi et al. [12].

2.6. BrdU-staining

5-Bromo-2′-deoxyuridine (BrdU, Sigma–Aldrich) at a dose of 75 mg/kg was injected i.p. 3 times at 2 h intervals at 4 weeks old, and the number of BrdU-

labeled cells in the hippocampus was counted at 8 weeks [12]. Sections were treated overnight with 0.1% Nonidet P-40/0.01 M PBS (pH 7.2) at 4 °C and denatured by microwave oven in 0.01 M citrate buffer (pH 6.0). After blocking in 10% goat serum/PBS with 0.1% NP-40 for 30 min, BrdU-positive cells in the sections were detected using a BrdU labeling and detection kit 2 (Roche Diagnostics GmbH, Germany) according to the manufacture's instructions.

2.7. Ki67 and parvalbumin staining

Sections were incubated with 10% goat serum/PBS with 0.1% Triton X-100, and then incubated with rabbit anti-Ki67 antibody (1:2000; Novocastra, Newcastle, UK) or rabbit anti-parvalbumin antibody (1:500; Calbiochem, San Diego, CA) overnight at 4 °C. They were washed with 0.01 M PBS and incubated with biotinylated goat anti-rabbit antibody (1:200; BA-1000, Vector Laboratories, Burlingame) at room temperature for 1 h. The sections were washed and processed with avidin-biotinylated horseradish peroxidase complex (Vector ABC kit, Vector Laboratories), and action was visualized using diaminobenzidine.

For double staining of parvalbumin/Nissl in the mPFC, sections were incubated with blocking solution (10% donkey serum/PBS with 0.1% Triton X-100) and then rabbit anti-parvalbumin antibody (1:500; Calbiochem) diluted in blocking solution was applied to sections, which were then incubated overnight at 4 °C and for 30 min at room temperature. After washing in PBS, donkey anti-rabbit Alexa 488 (1:1000; Invitrogen, Eugene, OR) was added to sections for 2 h at room temperature. NeuroTracer Fluorescent Nissl Stains (1:100; Invitrogen) diluted in PBS was applied to sections, which were then incubated for 20 min at room temperature.

2.8. Quantification for BrdU, Ki67 and parvalbmin-positive cells

All BrdU and Ki67-labeled cells in the subgranular zone (SGZ), hilus and granule cell layer (GCL) were assessed as described by Ibi et al. [12]. Parvalbumin-positive cells in the hippocampus and mPFC were counted under ×10 magnification using a light microscope (Axio Imager; Zeiss, Jene, Germany) and confocal-laser scanning microscope (LSM 510; Zeiss), respectively.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) followed by the Bonferroni test was used for multiple-group comparisons. Two-tailed Student's *t*-test was used for two-group comparisons.

3. Results

3.1. Behavioral evidence of gene–environment interactions

Neonatal WT and DN-DISC1 mice were treated with polyI:C or saline, and their behaviors were analyzed after being 8 weeks old. In the Y-maze test, neither genetic manipulation (vehicle/DN-DISC1) nor an environmental factor (polyI:C/WT) affected behavioral phenotypes compared with controls (vehicle/WT). Nonetheless, polyI:C/DN-DISC1 mice showed marked reduction in spontaneous

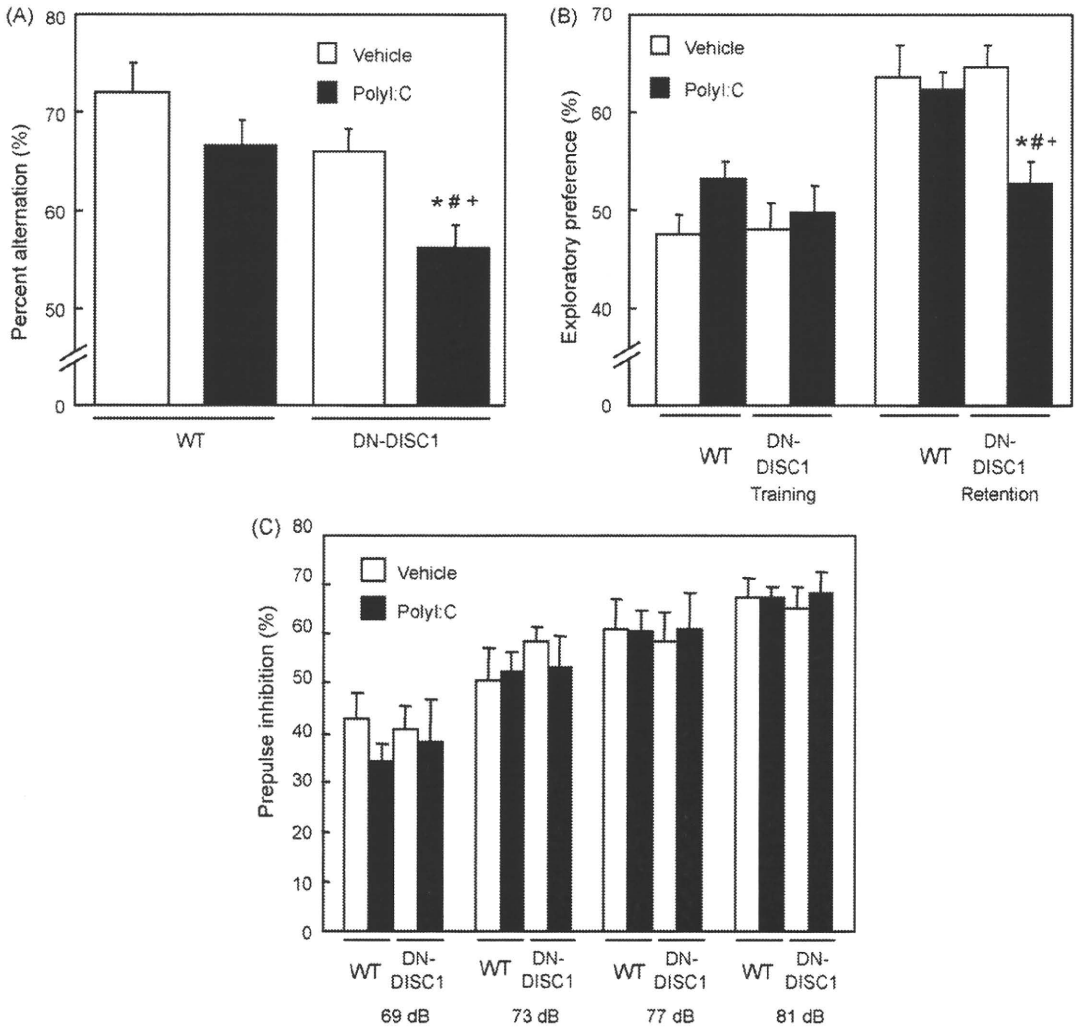

Fig. 1. Changes in short-term and recognition memories, and prepulse inhibition in polyI:C/DN-DISC1 mice. (A) Spontaneous alternation behavior in Y-maze test. Vehicle/WT, $n = 13$; polyI:C/WT, $n = 11$; vehicle/DN-DISC1, $n = 17$; polyI:C/DN-DISC1, $n = 13$. Percent alternation (%): $F_{(3,50)} = 6.529$, $p < 0.001$. (B) Exploratory preference in novel object recognition test. Vehicle/WT, $n = 12$; polyI:C/WT, $n = 11$; vehicle/DN-DISC1, $n = 10$; polyI:C/DN-DISC1, $n = 14$. Exploratory preference (%): $F_{(3,43)} = 5.427$, $p < 0.01$. (C) PPI (%) at four different prepulse intensities (69, 73, 77 and 81 dB) in PPI test. Vehicle/WT, $n = 9$; polyI:C/WT, $n = 14$; vehicle/DN-DISC1, $n = 11$; polyI:C/DN-DISC1, $n = 9$. Prepulse inhibition (%): $F_{(3,42)} = 0.0562$, $p = 0.982$. Values indicate the mean ± SE. $^{*}p < 0.05$ vs. vehicle/WT, $^{\#}p < 0.05$ vs. polyI:C/WT, $^{+}p < 0.05$ vs. vehicle/DN-DISC1.

alternation (Fig. 1A). There was no difference in the total number of arm entries among the four groups [vehicle/WT, 21.1 ± 1.0; polyI:C/WT, 24.7 ± 2.8; vehicle/DN-DISC1, 21.1 ± 0.9; polyI:C/DN-DISC1, 23.4 ± 1.7]. These results indicate a combined action of genetic (DN-DISC1) and environmental (polyI:C) factors, resulting in an impairment of short-term memory.

Similar effects were also observed as a marked decrease in exploratory preference to a novel object in the retention session of novel object recognition test, in which only polyI:C/DN-DISC1 mice displayed the deficit, whereas the other three groups showed no difference (Fig. 1B). There was no difference in total time exploring two objects among the four groups in the training [vehicle/WT, 30.0 ± 2.4 s; polyI:C/WT, 33.6 ± 3.2 s; vehicle/DN-DISC1, 33.6 ± 3.8 s; polyI:C/DN-DISC1, 33.0 ± 2.8 s]. Thus, polyI:C/DN-DISC1 mice have an impairment of object recognition memory.

On the contrary, in the PPI test of the startle reflex, no changes were observed in either polyI:C/WT, vehicle/DN-DISC1 or polyI:C/DN-DISC1 mice compared to vehicle/WT mice, indicating no impairment of sensorimotor gating in polyI:C/DN-DISC1 mice (Fig. 1C). There was also no difference in the acoustic startle amplitude [vehicle/WT, 215 ± 36; polyI:C/WT, 232 ± 36; vehicle/DN-DISC1, 162 ± 30; polyI:C/DN-DISC1, 184 ± 28].

We then focused on the characterization of schizophrenia-like behavioral abnormality in polyI:C/DN-DISC1 mice. Thus, in the following behavioral assays, behavioral changes in polyI:C/DN-DISC1 mice were compared with those in vehicle/WT mice without testing behaviors in polyI:C/WT or vehicle/DN-DISC1 mice.

In the fear conditioning memory test, polyI:C/DN-DISC1 mice showed a significant decrease in context-dependent freezing time compared to vehicle/WT mice (Fig. 2A). However, there was no difference in tone-dependent freezing time (35.5 ± 2.1 s in vehicle/WT mice, 33.0 ± 2.3 s in polyI:C/DN-DISC1 mice) or sensitivity to electric footshock (0.14 ± 0.02 mA in vehicle/WT mice, 0.15 ± 0.02 mA in polyI:C/DN-DISC1mice), suggesting an impairment of hippocampus-dependent fear memory in polyI:C/DN-DISC1mice.

In the social interaction test, time of interaction was markedly decreased, while escape and aggressive behaviors were increased, in polyI:C/DN-DISC1 mice, compared with vehicle/WT mice (Fig. 2B). MK-801-induced hyperactivity was significantly augmented in polyI:C/DN-DISC1 mice, compared to vehicle/WT mice (Fig. 2C).

3.2. Histological analyses in polyI:C/DN-DISC1 mice

Selective reduction in the immunoreactivity of parvalbumin, an indicator of a set of interneurons in the cerebral cortex, has frequently been reported in autopsied brains from patients with schizophrenia [17]. Very interestingly, polyI:C treatment in the neonatal stage developed the selective reduction in the immunoreactivity of parvalbumin, specific to the mPFC in DN-DISC1 mice (Fig. 3A and B).

Because polyI:C/DN-DISC1 mice showed a disturbance of the hippocampus-dependent fear memory, we examined whether there were histological alterations in the hippocampus of polyI:C/DN-DISC1 mice. We conducted Nissl-staining, BrdU staining, and immunohistochemistry for Ki67 (a marker of cell proliferation) [2,8]. Although there were no changes in Nissl-staining (data not shown) and immunostaining of Ki67 (Fig. 4A and B), a significant increase in the number of BrdU-positive cells in the GCL of the hippocampus was observed (Fig. 4A and C).

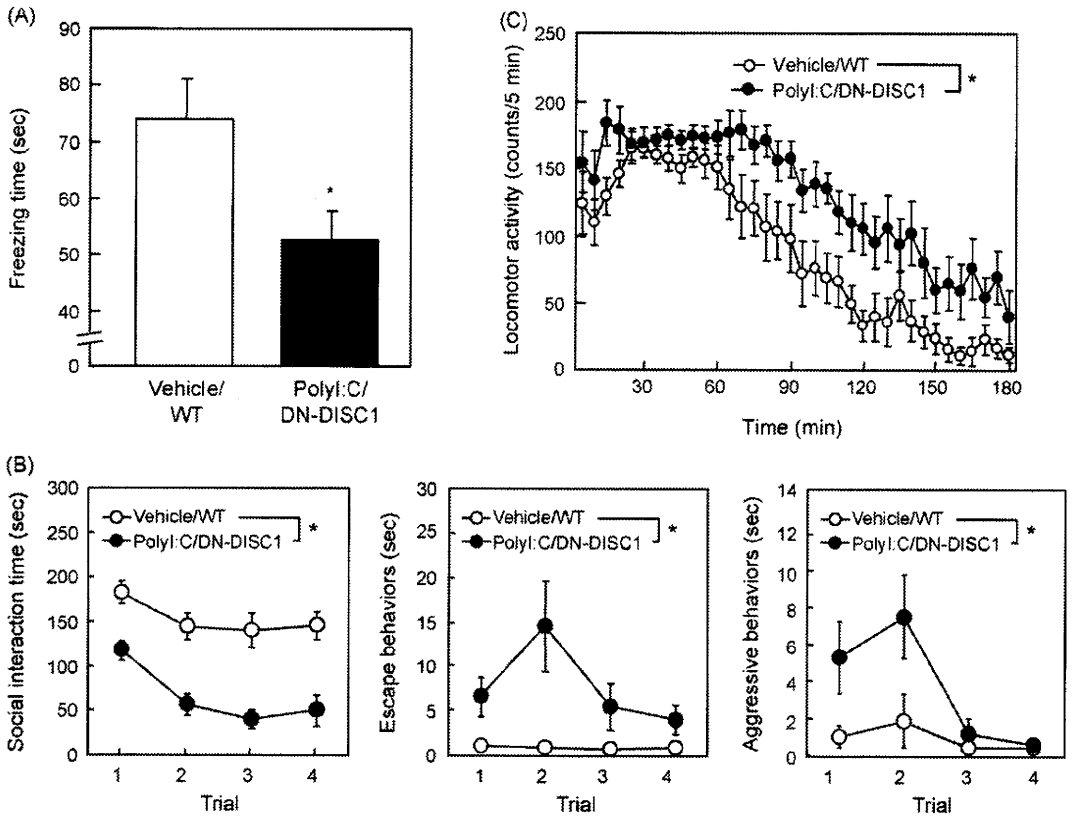

Fig. 2. Changes in fear memory, social behaviors and MK801-induced hyperactivity in polyI:C/DN-DISC1 mice. (A) Context-dependent memory in fear conditioning test. Vehicle/WT, $n = 11$; polyI:C/DN-DISC1, $n = 13$. (B) Social behaviors in social interaction test. Vehicle/WT, $n = 8$; polyI:C/DN-DISC1, $n = 8$. Social interaction time: $F_{(1,14)} = 41.172$; $p < 0.0001$, escape behaviors: $F_{(1,14)} = 7.012$; $p < 0.05$, aggressive behaviors: $F_{(1,14)} = 7.316$; $p < 0.05$. (C) MK-801-induced hyperactivity. Vehicle/WT, $n = 9$; polyI:C/DN-DISC1, $n = 10$. $F_{(1,17)} = 11.232$, $p < 0.01$. Values indicate the mean ± SE. *$p < 0.05$ vs. vehicle/WT.

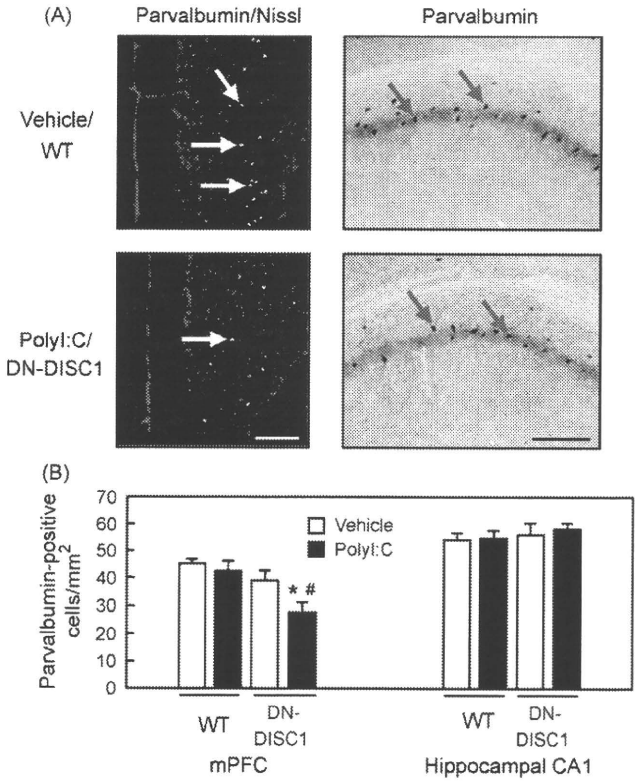

Fig. 3. Changes in parvalbumin-positive interneurons in polyI:C/DN-DISC1 mice. (A) Representative photographs showing parvalbumin-positive cells in the mPFC [left (parvalbumin-positive cell, green; Nissl-positive cell, red)] and hippocampal CA1 region (right). Upper panels, vehicle/WT mice; lower panels, polyI:C/DN-DISC1 mice. Scale bar: 200 μm. (B) Changes in the number of parvalbumin-positive interneurons. mPFC: vehicle/WT, $n = 10$; polyI:C/WT, $n = 12$; vehicle/DN-DISC1, $n = 8$; polyI:C/DN-DISC1, $n = 8$. $F_{(3,35)} = 4.996$, $p < 0.001$. Hippocampal CA1 region: vehicle/WT, $n = 3$; polyI:C/WT, $n = 4$; vehicle/DN-DISC1, $n = 3$; polyI:C/DN-DISC1, $n = 4$. $F_{(3,10)} = 0.386$, $p = 0.766$. Values indicate the mean ± SE. $^{*}p < 0.05$ vs. vehicle/WT, $^{\#}p < 0.05$ vs. polyI:C/WT.

4. Discussion

In agreement with the previous report by Hikida et al. [10], behavioral and histological abnormalities in DN-DISC1 mice were mild and subtle. In the present study, we demonstrated that combination of neonatal polyI:C treatment with DN-DISC1 resulted in the deficits of short-term, object recognition, and hippocampus-dependent fear memories in adulthood, although polyI:C treatment by itself had little influence on WT mice. Furthermore, polyI:C/DN-DISC1 mice exhibited signs of impairment of social recognition and interaction and augmented susceptibility to MK-801-induced hyperactivity. DN-DISC1 mice is reported to display a small, but significant, deficit in PPI of the startle reflex in the previous study [10], whereas there was no significant difference between vehicle/WT and vehicle/DN-DISC1 mice in the present study. The discrepancy in the PPI deficit of DN-DISC1 mice may be explained by the difference in experimental schedule. Behavioral analysis was carried out at the age of 12–32 weeks in the previous report [10] while we started the analysis from 8-week old mice. Accordingly, it is possible that the changes in PPI response in DN-DISC1 mice may be undetectable at younger ages as we selected [24].

The present study demonstrated a significant impact of DN-DISC1 expression on parvalbumin-positive interneurons in the mPFC (Fig. 3A and B), which is consistent with the previous findings [10]. Importantly, the post hoc analysis revealed the additive effect of neonatal immune activation induced by polyI:C treatment and genetic impact of DN-DISC1, leading to a marked decrease in parvalbumin-positive interneurons in the mPFC of polyI:C/DN-DISC1 mice in adulthood. This pathological change is currently regarded as the best hallmark for the pathophysiology of schizophrenia, which is likely to underlie the cognitive dysfunction in patients with schizophrenia [17,20].

For the past couple of years, many lines of genetic mouse models based on susceptibility genes for schizophrenia have become available and characterized mainly by behavioral alterations [10,13,18,19,25]. Nonetheless, only a few studies have addressed for possible gene-environmental interactions in the context of schizophrenia [16]. We believe that the present study has two major strengths: first, a most promising pair of genetic and environmental factors for schizophrenia is tested together for the phenotypic assessment; second, of most importance, the present study addresses a specific interneuron deficit, well-accepted histological hallmark for schizophrenia, and examine how gene-environmental interactions during the "critical neurodevelopmental period" can result in this objective phenotype in "adulthood". Furthermore, we also demonstrated that such gene-environmental interactions specifically affect some, but not all, types of behaviors elicited by the genetic factor. The concept of neonatal "critical neurodevelopmental period" proposed in the

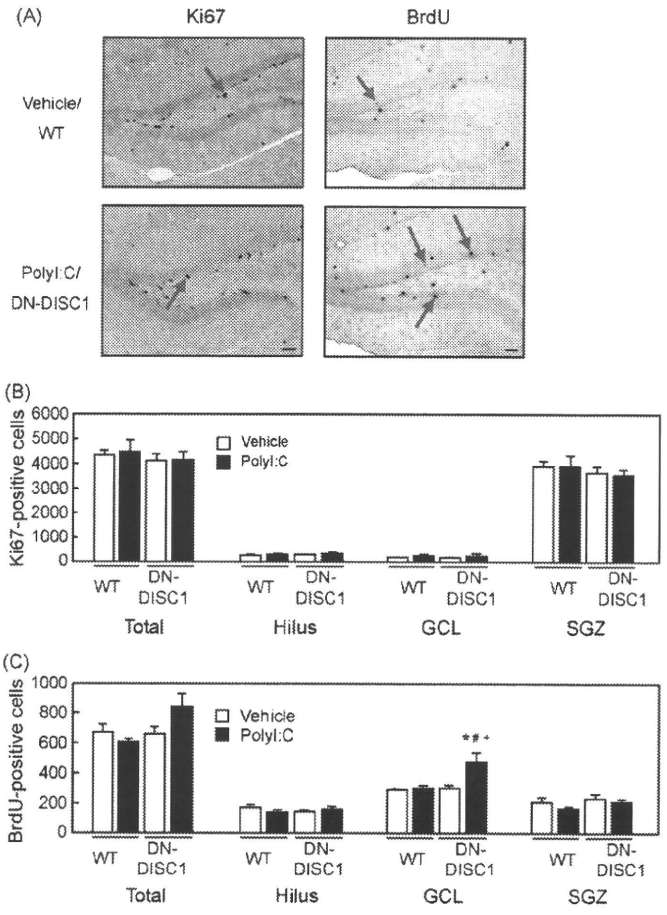

Fig. 4. Changes in Ki67 and BrdU-positive cells in polyI:C/DN-DISC1 mice. (A) Representative photographs showing Ki67-positive, and BrdU-positive cells in the DG of hippocampus. Upper panels, vehicle/WT mice; lower panels, polyI:C/DN-DISC1 mice. Scale bar: 200 μm. (B) Changes in the number of Ki67-positive cells in the hippocampus. Vehicle/WT, $n = 3$; polyI:C/WT, $n = 4$; vehicle/DN-DISC1, $n = 3$; polyI:C/DN-DISC1, $n = 4$. Total: $F_{(3,10)} = 0.231$, $p = 0.873$, Hilus: $F_{(3,10)} = 0.318$, $p = 0.812$, GCL: $F_{(3,10)} = 0.926$, $p = 0.463$, SGZ: $F_{(3,10)} = 0.358$, $p = 0.785$. (C) Changes in the number of BrdU-positive cells in the hippocampus. Vehicle/WT, $n = 5$; polyI:C/WT, $n = 5$; vehicle/DN-DISC1, $n = 5$; polyI:C/DN-DISC1, $n = 5$. $F_{(3,16)} = 6.406$, $p < 0.01$. There were no significant differences in other brain areas. Values indicate the mean ± SE. $^{*}p < 0.05$ vs. vehicle/WT, $^{\#}p < 0.05$ vs. polyI:C/WT, $^{+}p < 0.05$ vs. vehicle/DN-DISC1.

present study is consistent with the observation by the Cannon and Silva's group [19], in which their group demonstrated the critical requirement of DISC1 function in neonatal days by using inducible transgenic mice for mutant DISC1. On the other hand, from the clinical point of view, "critical development period" of schizophrenia is postulated not only in early (pre- and peri-natal) stage but also in late (pubertal) stage in human [26]. Further studies are required to clarify the effect of pubertal exposure to psychosocial stresses or polyI:C in DN-DISC1 mice on phenotypic changes.

To elucidate a genuine gene-based effect, it is important to use more than two independent lines of transgenic mice. The present study investigated the gene-environmental interaction by using only one line of DN-DISC1 mice (line 10) because basic characterization of these behavioral deficits has already conducted in two independent lines (lines 10 and 37) which showed that the behavioral and histological phenotypes in DN-DISC1 mice are due to the disruption of DISC1 gene [10]. However, to completely exclude a possibility that another, unknown gene may participate in the interaction, we need to use another line of DN-DISC1 (line 37).

In summary, taking effects in several behavioral and pathophysiological deficits into consideration, we propose that neonatal polyI:C treatment in DN-DISC1 mice may provide a model for schizophrenia that reflects gene-environmental interactions.

Conflict of interest

The authors declare that there is no conflict of interest in the publication of the present work.

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$GABA_B$ receptor agonist baclofen improves methamphetamine-induced cognitive deficit in mice

Sawako Arai [a,1], Kazuhiro Takuma [a,1], Hiroyuki Mizoguchi [a,b], Daisuke Ibi [a], Taku Nagai [c], Hiroyuki Kamei [d], Hyoung-Chun Kim [e], Kiyofumi Yamada [a,c,f,*]

[a] Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan
[b] Futuristic Environmental Simulation Center, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan
[c] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
[d] Laboratory of Clinical Pharmacy Practice and Health Care Management, Faculty of Pharmacy, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya 468-8503, Japan
[e] Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200-701, South Korea
[f] CREST, JST, Nagoya 466-8560, Japan

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ABSTRACT

In this study, we investigated the effects of $GABA_A$ and $GABA_B$ receptor agonists on the methamphetamine-induced impairment of recognition memory in mice. Repeated treatment with methamphetamine at a dose of 1 mg/kg for 7 days induced an impairment of recognition memory. Baclofen, a $GABA_B$ receptor agonist, ameliorated the repeated methamphetamine-induced cognitive impairment, although gaboxadol, a $GABA_A$ receptor agonist, had no significant effect. $GABA_B$ receptors may constitute a putative new target in treating cognitive deficits in patients suffering from schizophrenia, as well as methamphetamine psychosis.

1. Introduction

Methamphetamine is a highly addictive drug of abuse, and addiction to methamphetamine has increased to epidemic proportions worldwide (Cretzmeyer et al., 2003; Rawson et al., 2002). Chronic use of methamphetamine causes psychiatric symptoms, such as hallucination and delusions, and long-term cognitive deficits (Simon et al., 2000; Kalechstein et al., 2003; Nordahl et al., 2003; Srisurapanont et al., 2003), which are indistinguishable from paranoid schizophrenia (Yui et al., 2002; Srisurapanont et al., 2003). In a previous study, we demonstrated that repeated methamphetamine treatment caused an enduring impairment of recognition memory in a novel object recognition test in mice, and that methamphetamine-induced cognitive impairment was reversed by an atypical antipsychotic, clozapine, but not haloperidol (Kamei et al., 2006). Furthermore, the same treatment in rats resulted in a significant impairment of spatial working memory, which was ameliorated by clozapine but not haloperidol (Nagai et al., 2007). Thus, methamphetamine-induced memory impairment in rodents may be a useful model for cognitive deficits in methamphetamine abusers and schizophrenic patients.

The GABA receptor system is known to play a significant role in modulating the dopamine system (Tepper and Lee, 2007). Several studies have demonstrated that GABA receptor agonists can inhibit the effects of drugs of abuse. For example, baclofen has been shown to attenuate amphetamine-induced increase in dopamine levels in the nucleus accumbens (Brebner et al., 2005), and $GABA_A$ receptors on ventral tegmental area dopamine neurons play a significant role in attenuating the effects of drugs of abuse in a similar manner to that of $GABA_B$ receptors (Westerink et al., 1996). Although many studies have examined the effects of GABA receptor agonists on hyperdopaminergic conditions induced by psychostimulant drugs, few studies have investigated the effects of GABA receptors on cognitive deficits induced by drugs of abuse.

Recent studies suggest that alterations of GABA systems are related to the pathophysiology of schizophrenia (Lewis, 2000; Benes and Berretta, 2001). Moreover, it is suggested that impairment in GABA-mediated inhibition in the prefrontal cortex may provide a mechanism of disturbance in cognitive processes, such as working memory, in individuals with schizophrenia (Lewis, 2000; Benes and Berretta, 2001). Cognitive dysfunction is considered a core feature of schizophrenia (Elvevåg and Goldberg, 2000), and the degree of cognitive deficit may be the best predictor of long-term functional outcome for individuals with schizophrenia (Green, 1996). Despite the clinical

* Corresponding author. Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan. Tel.: +81 52 744 2674; fax: +81 52 744 2682.
E-mail address: kyamada@med.nagoya-u.ac.jp (K. Yamada).
[1] S.A. and K.T. contributed equally to this work.

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importance of cognitive dysfunction in schizophrenia, there are no appropriate drug therapies.

In this study, to develop novel pharmacotherapy for cognitive deficits in schizophrenia patients and methamphetamine abusers, we examined the effects of $GABA_A$ and $GABA_B$ receptor agonists on methamphetamine-induced impairment of recognition memory in mice.

2. Materials and methods

Male ICR mice (7–8 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). 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 animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Methamphetamine hydrochloride (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), *R*(+)-baclofen hydrochloride (Sigma-Aldrich Co., St Louis, MO) and gaboxadol hydrochloride (Sigma-Aldrich) were dissolved in saline. All drugs were administered in a volume of 0.1 ml/10 g body weight. Mice were given methamphetamine (1 mg/kg, s.c.) daily once for 7 days. One day after the last treatment of methamphetamine, novel object recognition test commenced as described below.

Novel object recognition test was carried out as described previously (Kamei et al., 2006; Mizoguchi et al., 2008). The experimental apparatus consisted of a Plexiglas box (30×30×35 cm high), with a sawdust-covered floor. The apparatus was located in a sound-attenuated room and was illuminated with a 20 W bulb.

The novel object recognition test procedure consisted of three sessions: habituation, training, and retention. Each mouse 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 a golf ball, a wooden column and a wall socket, which were different in shape and color but similar in size. The animals were considered to be exploring the object when the head of the animal was facing the object or the animal was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions, the animals were placed back into the same box 24 h (day 5) after the training session, in which one of the familiar objects used during training was replaced by 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, the 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 a ratio of the time spent exploring the object that was replaced by the novel object in the retention session, over the total exploring time.

Baclofen (1 and 2 mg/kg, s.c.) and gaboxadol (1 and 3 mg/kg, s.c.) were administered once 15 min before the training session in novel object recognition test (day 4). No drugs were given during the habituation (day 1–3) and the retention sessions (day 5) in the novel object recognition test.

To investigate effect of baclofen on motor function, locomotor activity was measured. Mice were given saline or methamphetamine at a dose of 1 mg/kg for 7 days. Mice were placed in home cage for 15 min following injection of saline or baclofen (2 mg/kg, s.c.) after the 3 day-withdrawal of repeated methamphetamine treatment, and then locomotor activity was measured for 10 min in a standard transparent rectangular rodent cage (25×30×18 high cm) using an infrared sensor (NS-AS01; Brainscience, Osaka, Japan) placed over the cage (Kamei et al., 2006; Mizoguchi et al., 2008).

All data were expressed as the mean±S.E.M.. Statistical analysis was carried out by one-way or two-way ANOVA, followed by Student–Newman–Keuls test for multigroup comparisons. *P* values less than 0.05 were taken to indicate significant differences.

3. Results

Repeated methamphetamine treatment (1 mg/kg, s.c.) for 7 days resulted in a significant reduction of the preference index in the retention session but not training session as compared with saline-treated control (Figs. 1A and 2A) although it had no effect on total exploratory time (Figs. 1B and 2B). The $GABA_A$ receptor agonist, gaboxadol, at doses of 1 mg/kg, failed to ameliorate the methamphetamine-induced reduction of exploratory preference to the novel object in the retention session of novel object recognition test (Fig. 1A). Although there was no difference between gaboxadol 1 mg/kg and 3 mg/kg in methamphetamine-treated animal ($P=0.10$), there was a tendency of recovery in gaboxadol-treated group at 3 mg/kg ($P=0.06$). Thus, we

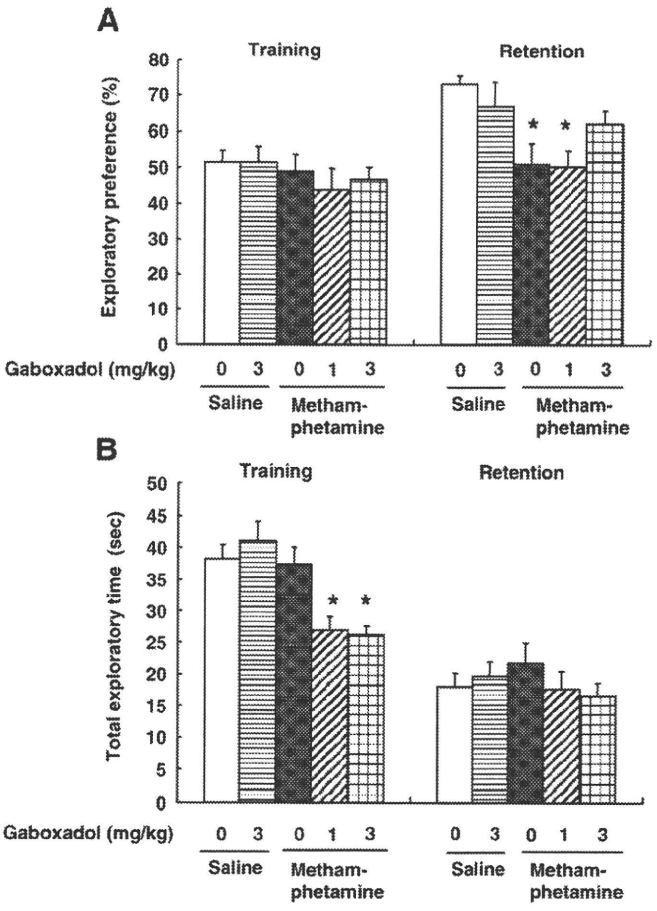

Fig. 1. Effect of gaboxadol on methamphetamine-induced impairment of recognition memory in mice. After the cessation of repeated methamphetamine (1 mg/kg, s.c.) treatment for 7 days, mice were subjected to the novel-object recognition test. Gaboxadol (1 and 3 mg/kg, s.c.), or saline was administered 15 min before the training session. (A) Exploratory preference. (B) Total exploration time. Values indicate the mean±S.E.M. (saline/saline, $n=13$; saline/Gaboxadol 3 mg/kg, $n=8$; methamphetamine/saline, $n=12$; methamphetamine/Gaboxadol 1 mg/kg, $n=7$; methamphetamine/Gaboxadol 3 mg/kg, $n=14$). ANOVA: (A, training) $F(4,49)=0.488$, $P=0.7448$; (A, retention) $F(4,49)=4.6$, $P<0.01$; (B, training) $F(4,49)=7.876$, $P<0.01$; (B, retention) $F(4,49)=0.637$, $P=0.6389$. $^*P<0.05$ compared with the saline/saline-treated group (Student–Newman–Keuls test).

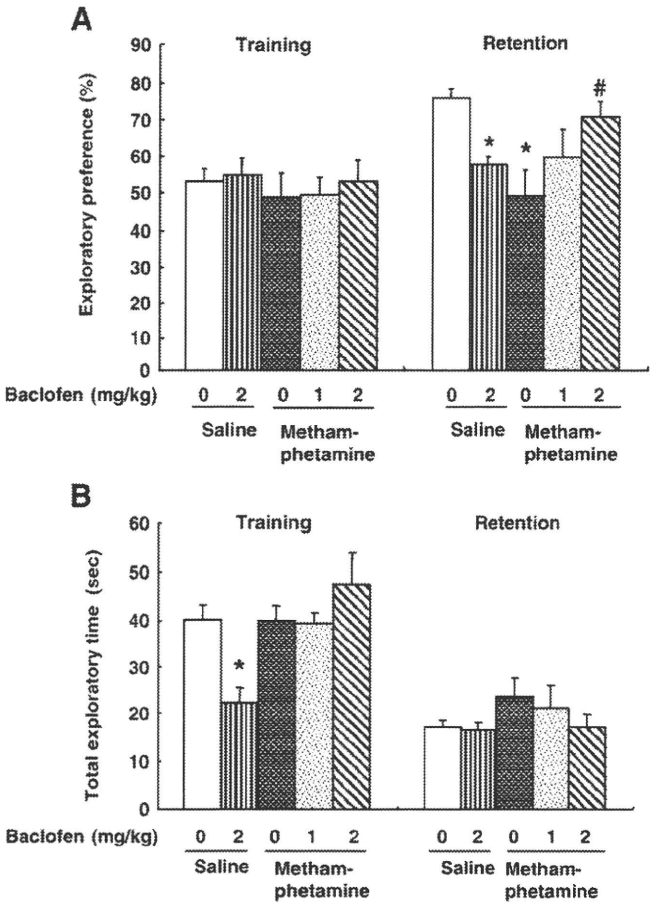

Fig. 2. Effect of baclofen on methamphetamine-induced impairment of recognition memory in mice. After the cessation of repeated methamphetamine (1 mg/kg) treatment for 7 days, mice were subjected to the novel-object recognition test. Baclofen (1 and 2 mg/kg, s.c.) or saline was administered 15 min before the training session. (A) Exploratory preference. (B) Total exploration time. Values indicate the mean ± S.E.M. (saline/saline, $n=12$; saline/Baclofen 2 mg/kg, $n=13$; methamphetamine/saline, $n=8$; methamphetamine/Baclofen 1 mg/kg, $n=8$; methamphetamine/Baclofen 2 mg/kg, $n=10$). ANOVA: (A, training) $F(4,46)=0.242$, $P=0.9133$; (A, retention) $F(4,46)=5.56$, $P<0.01$; (B, training) $F(4,46)=7.752$, $P<0.01$; (B, retention) $F(4,46)=1.2$, $P=0.3238$. $^{*}P<0.05$ compared with the saline/saline-treated group. $^{\#}P<0.05$ compared with the methamphetamine/saline-treated group (Student–Newman–Keuls test).

also examined the effect of gaboxadol at 10 mg/kg. However, because high-dose gaboxadol at 10 mg/kg markedly reduced the exploratory activity of mice in the training session, they were not subjected to novel object recognition test (data not shown). Gaboxadol at 3 mg/kg had no effect on the exploratory preference (Fig. 1A) and total exploratory time (Fig. 1B) in both training and retention sessions in saline-treated control mice.

Next, we examined the effect of baclofen on methamphetamine-induced cognitive impairment. The $GABA_B$ receptor agonist dose-dependently improved the reduction of exploratory preference to the novel object in methamphetamine-treated mice (Fig. 2A). Baclofen at 2 mg/kg significantly ameliorated methamphetamine-induced cognitive impairment (Fig. 2A). Baclofen had no effect on the level of exploratory preference for the novel object in the training session or the total exploration time in both the training and retention sessions in methamphetamine-treated mice. Treatment with baclofen at 2 mg/kg in saline-treated control group resulted in a significant decrease in total exploratory time to novel objects in the training session (Fig. 2B), leading to a significant impairment of novel object recognition in the retention session (Fig. 2A). This is probably due to an insufficient exploratory behaviors in the training session, which could result in a poor discrimination of a novel object.

4. Discussion

We have previously demonstrated that repeated methamphetamine treatment in mice induces enduring recognition memory impairment, which is associated with dysfunction of the dopamine D_1 receptor-ERK1/2 pathway in the prefrontal cortex. Clozapine, but not haloperidol, completely restored the cognitive impairment induced by methamphetamine treatment when repeatedly administered for 7 days after withdrawal from methamphetamine, although acute treatment with these antipsychotics had no effect (Kamei et al., 2006). The data are consistent with clinical evidence that clozapine is superior to typical neuroleptics in improving cognitive deficits in schizophrenic patients (Lee et al., 1999). Thus, we propose that methamphetamine-induced cognitive impairment in mice may be a useful model for cognitive deficits in methamphetamine abusers and schizophrenic patients. In this study, we found that acute treatment with baclofen improved methamphetamine-induced cognitive deficit without affecting motor function, whereas repeated treatment was necessary for the effect of clozapine. These results suggest that $GABA_B$ receptor agonists may be more useful for the treatment of cognitive deficit in schizophrenia patients and methamphetamine abusers than clozapine and other antipsychotic drugs. In contrast, gaboxadol, a $GABA_A$ receptor agonist, had no effect on methamphetamine-induced cognitive deficits. However, gaboxadol is known to preferentially activate the $GABA_A$ receptor subtype containing the delta subunit, which mediated tonic inhibition. Therefore, gaboxadol may not be an ideal agonist for a global activation of $GABA_A$ receptors. Further studies are required to test this assumption.

Additionally, we think that the ameliorating effect of baclofen is not related to the effect on motor function. In fact, we examined the effect of baclofen at a dose of 2 mg/kg on locomotor activity of mice that had been treated with saline or methamphetamine (1 mg/kg) for 7 days. Baclofen had no effect on behavioral locomotion of repeated methamphetamine-treated group (saline/saline group ($n=7$), 399 ± 39.9 counts/10 min; saline/baclofen group ($n=7$), 343.7 ± 51.4 counts/10 min; methamphetamine/saline group ($n=7$), 429.1 ± 21.4 counts/10 min; methamphetamine/baclofen group ($n=7$), 346.3 ± 41.6 counts/10 min; $F(3,24)=1.08$, $P=0.37$). In Figs. 1 and 2, we showed the total exploratory time, which means locomotor activity in training and retention phase, respectively. Baclofen had no effect on the level of exploratory preference for the novel object in the training session or the total exploration time in both the training and retention sessions in methamphetamine-treated mice. These results suggest that baclofen has no effect on motor function in methamphetamine-treated mice. There was an apparent difference in sensitivity to baclofen between saline-treated control and methamphetamine-treated group: Baclofen at 2 mg/kg significantly reduced the total exploratory time in the training session in control mice, while the drug had no effect in the methamphetamine-treated mice. Regarding to this phenomenon, it is reported that repeated cocaine treatment decreases baclofen-stimulated [^{35}S]GTPγS binding to G protein in the nucleus accumbens, indicating desensitization of $GABA_B$ receptors (Xi et al., 2003). Thus, it is possibly that repeated methamphetamine treatment causes desensitization of $GABA_B$ receptor as does cocaine treatment.

There are some studies suggesting that $GABA_B$ receptors play an important role in regulating dopamine neurons, while the role of $GABA_A$ receptors has been unclear. For example, previous studies showed that baclofen reduced the reinforcing effects of many substances of abuse, such as cocaine, nicotine, heroin, and alcohol (Cousins et al., 2002), possibly through $GABA_B$-mediated modulation of mesolimbic dopamine transmission (Bartholini, 1985). In fact, baclofen is known to stabilize the firing pattern of dopamine neurons (Erhardt et al., 2002). It was demonstrated that chronic coadministration of baclofen and amphetamine blocked the development of sensitization to the locomotor stimulation effect of amphetamine

(Bartoletti et al., 2005), and acute treatment with baclofen inhibited the expression of amphetamine-induced locomotor sensitization (Bartoletti et al., 2004). Moreover, a recent study showed that acute treatment with baclofen ameliorated ethanol-induced memory deficit in mice (Escher and Mittleman, 2004). Moreover, we have recently demonstrated that baclofen, but not gaboxadol, ameliorates methamphetamine- and MK-801-induced impairment of prepulse inhibition of the acoustic startle reflex in mice (Arai et al., 2008). These results support our findings that baclofen ameliorates repeated methamphetamine treatment-induced cognitive deficits. Taken together, the ameliorating effect of baclofen on cognitive impairment in methamphetamine-treated mice may be attributable to its effects on $GABA_B$ receptors in midbrain dopamine neurons.

In conclusion, we demonstrated that baclofen acutely ameliorated the cognitive deficit in repeated methamphetamine-treated mice, an animal model for cognitive deficits in methamphetamine abuse and schizophrenia. Our results suggest that baclofen may be superior to clozapine and other antipsychotic drugs that mainly affect dopamine D_2 and 5-HT_2 receptors. $GABA_B$ receptors may constitute a putative new target for treating cognitive deficits in patients suffering from schizophrenia, as well as methamphetamine psychosis. Further studies are necessary to clarify the molecular mechanisms of the action of baclofen.

Conflict of interest

There are no conflicts of interest in this study.

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Dysfunction of dopamine release in the prefrontal cortex of dysbindin deficient sandy mice: An *in vivo* microdialysis study

Taku Nagai[a,1], Yuko Kitahara[a,1], Anna Shiraki[a], Takao Hikita[b], Shinichiro Taya[b], Kozo Kaibuchi[b,c], Kiyofumi Yamada[a,c,*]

[a] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
[b] Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
[c] CREST, JST, Nagoya 466-8560, Japan

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ABSTRACT

Dystrobrevin binding protein-1 gene (*DTNBP1*), which encodes dysbindin protein, has been identified as a schizophrenia susceptibility gene. Dysbindin has been shown to contribute to the regulation of exocytosis and formation of synaptic vesicles. Although hypofrontality in schizophrenia underlies its pathophysiology, the molecular function of dysbindin in synaptic neurotransmission remains unclear. In the present study, we investigated depolarization-evoked dopamine (DA) and serotonin (5-HT) release in the prefrontal cortex (PFC) of sandy (sdy) mice, which have a deletion mutation in the gene encoding *DTNBP1*. *In vivo* microdialysis analysis revealed that extracellular DA levels in the PFC of wild-type mice were increased by 60 mM KCl stimulation, and the KCl-evoked DA release was significantly decreased in sdy mice compared with wild-type mice. Extracellular 5-HT levels in the PFC of wild-type mice were also increased by 60 mM KCl stimulation. The KCl-evoked 5-HT release did not differ between wild-type and sdy mice. There was no difference in basal levels of DA and 5-HT before the stimulation between two groups. Behavioral sensitization after repeated methamphetamine (METH) treatment was significantly reduced in sdy mice compared with wild-type mice whereas no difference was observed in METH-induced hyperlocomotion between two groups. These results suggest that dysbindin may have a role in the regulation of depolarization-evoked DA release in the PFC and in the development of behavioral sensitization induced by repeated METH treatment.

Schizophrenia is a devastating psychiatric disorder with a prevalence of 0.5–1.0%. Clinical symptoms are categorized into positive symptoms, negative symptoms, and cognitive dysfunction. Previous studies have suggested that dysfunction of dopaminergic (DAergic), glutamatergic, or GABAergic neurotransmission underlies the pathophysiology of schizophrenia [17]. Especially, hypofunction of the mesocortical DAergic system may be related to negative symptoms and cognitive dysfunction whereas hyperfunction of the mesolimbic DAergic system causes positive symptoms [5]. Serotonergic system is also implicated in features of schizophrenia since serotonin (5-HT)–dopamine (DA) antagonism by atypical antipsychotics can improve some positive and negative symptoms [25].

Dystrobrevin binding protein-1 gene (*DTNBP1*), which encodes dysbindin protein, has been identified as a schizophrenic associated gene through linkage and association analysis [28]. Postmortem brain studies have demonstrated that expression of dysbindin protein and mRNA are reduced in the PFC and hippocampus of schizophrenia [32,33]. Moreover, it has been demonstrated that a risk haplotype of *DTNBP1* in schizophrenia is associated with negative symptoms in schizophrenia [7].

Many studies have indicated that dysbindin contributes to the regulation of exocytosis and/or formation of synaptic vesicles. Dysbindin concentrates in the fraction containing enriched synaptic vesicle membrane [31]. Hikita et al. [11] demonstrated that dysbindin partially colocalizes with synaptophysin in primary cultured hippocampal neuron. Knockdown of dysbindin has affected the extracellular glutamate or DA levels in PC12 cells [16,22]. In addition to these *in vitro* studies, sandy (sdy) mouse has been available to study the physiologic function of dysbindin. Sdy mice arose from spontaneous mutation in a DBA/2J stock and carry a *Dtnbp1* allele encoding a protein with an in-frame 22-residue deletion [18]. Behavioral analysis revealed that sdy mice display impairments of cognitive function [1,30] and exhibit anxiety-related behavior

* Corresponding author at: Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan. Tel.: +81 52 744 2674; fax: +81 52 744 2979.
E-mail address: kyamada@med.nagoya-u.ac.jp (K. Yamada).
[1] Contributed equally to the work.

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[10]. Sdy mice have lower tissue content of DA in the cortex and hippocampus [19]. However, it remains to be determined if the regulation of neurotransmitter release in the brains of sdy mice is altered. In the present study, to investigate the molecular function of dysbindin in synaptic neurotransmission, we measured extracellular DA and 5-HT levels in the PFC of sdy mouse before and after depolarization stimulus, using *in vivo* microdialysis. In addition, we analyzed methamphetamine (METH)-induced hyperlocomotion and behavioral sensitization in sdy mice.

Sdy mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The animals were housed in plastic cages and kept in a regulated environment (23 ± 1 °C, 50 ± 5% humidity), with a 12/12 h light–dark cycle (lights on at 9:00 a.m.). Food (CE2; Clea Japan Inc., Tokyo, Japan) and tap water were available ad libitum. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University.

For *in vivo* microdialysis, 11–18-week-old homozygous sdy mice ($n = 7$) and wild-type DBA/2J mice ($n = 6$) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a guide cannula (AG-4, Eicom Corp., Kyoto, Japan) was implanted in the PFC (+2.0 mm anteroposterior, −0.5 mm mediolateral from the bregma, −2.0 mm dorsoventral from the skull) according to the mouse brain atlas. On recovery from the surgery, a dialysis probe (A-I-4-01; membrane length 1 mm, Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF, 147 mmol/l NaCl, 4 mmol/l KCl, and 2.3 mmol/l $CaCl_2$) at a flow rate of 1.0 μl/min. The outflow fractions were collected every 10 min. After the collection of baseline fractions, high potassium-containing aCSF (60 mM; isomolar replacement of NaCl with KCl) was perfused for 20 min through the dialysis probe. DA and 5-HT levels in the dialysates were analyzed using an HPLC system (HTEC-500, Eicom Corp.) equipped with an electrochemical detector [20]. The probe recoveries of DA and 5-HT were approximately 25%.

For measurement of locomotor activity, mice were placed individually in a transparent acrylic cage with a black frosting Plexiglas floor (25 cm × 25 cm × 20 cm), and locomotor activity was measured every 5 min for 60 min using digital counters with an infrared sensor (BrainScience Idea, Osaka, Japan). Wild-type and sdy mice were habituated to the test environment for 120 min before the measurement of locomotor activity. They were injected with saline or METH (0.5 or 1.0 mg/kg, s.c.), and the locomotor activity was measured for 60 min. For the behavioral sensitization, mice were injected with METH (1.0 mg/kg, s.c.) once a day for 5 days, and METH-induced locomotor activity was measured for 60 min.

All data were expressed as the mean ± S.E. Statistical significance was determined using a two-way analysis of variance (ANOVA) with or without repeated measures followed by the Bonferroni test when F ratios were significant ($p < 0.05$).

First of all, we studied the effect of depolarization stimulus on DA release in the PFC of sdy mice by *in vivo* microdialysis. As shown in Fig. 1, extracellular DA levels in the PFC of wild-type mice were increased by 60 mM KCl stimulation, and the KCl-evoked DA release was significantly diminished in sdy mice compared to wild-type mice [an ANOVA with repeated measures, genotype, $F_{(1,11)} = 8.130$, $p < 0.05$; time after the KCl treatment, $F_{(5,55)} = 37.810$, $p < 0.01$; genotype by time after the KCl treatment interaction, $F_{(5,55)} = 2.267$, $p > 0.05$]. Post hoc analysis revealed that depolarization-evoked DA release was markedly decreased in sdy mice compared with wild-type mice from 10 to 50 min after 60 mM KCl treatment ($p < 0.05$, Fig. 1). There was no difference in the basal levels of DA before the stimulation between the two groups (wild-type, 0.19 ± 0.04 pg/10 μl/10 min; sdy, 0.17 ± 0.02 pg/10 μl/10 min).

The mesocorticolimbic system is responsible for the locomotor-stimulating effects of METH. Therefore, we measured the

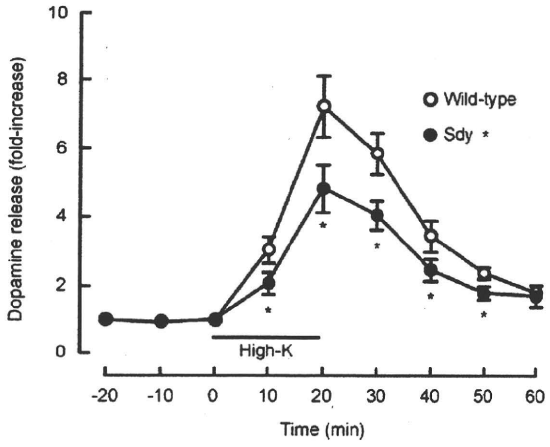

Fig. 1. Depolarization-evoked DA release in the PFC of sdy mice measured by *in vivo* microdialysis. A dialysis probe was inserted through the guide cannula, and perfused with an aCSF at a flow rate of 1.0 μl/min. Following the collection of three baseline fractions, high potassium-containing aCSF (60 mM KCl) was perfused for 20 min through the dialysis probe. Values represent the mean ± S.E. (wild-type, $n = 6$; sdy, $n = 7$). *$p < 0.05$ compared to the corresponding wild-type mice.

locomotor-stimulating effects of METH in sdy mice. Single METH (0.5 and 1.0 mg/kg s.c.) treatment induced hyperlocomotion in both wild-type and sdy mice [a two-way ANOVA, METH treatment, $F_{(2,42)} = 11.977$, $p < 0.01$, Fig. 2A]. There was no difference in the

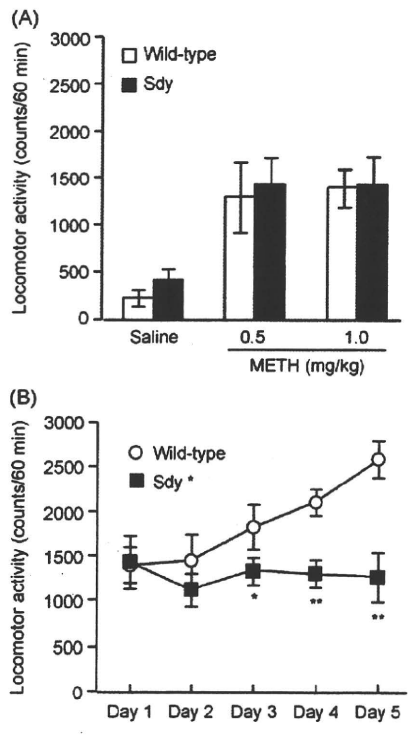

Fig. 2. METH-induced hyperlocomotion and behavioral sensitization in sdy mice. (A) Single METH-induced hyperlocomotion. Wild-type ($n = 7$ in each group) and sdy ($n = 9$ in each group) mice were habituated to the test environment for 120 min before the measurement of locomotor activity. Wild-type and sdy mice were injected with saline or methamphetamine (METH, 0.5 or 1.0 mg/kg, s.c.), and the locomotor activity was measured for 60 min. (B) Repeated METH-induced behavioral sensitization. Wild-type ($n = 7$) and sdy ($n = 9$) mice were habituated to the test environment for 120 min before the measurement of locomotor activity. Mice were injected with METH (1.0 mg/kg, s.c.) once a day for 5 days, and METH-induced locomotor activity was measured for 60 min. Values represent the mean ± S.E. *$p < 0.05$ and **$p < 0.01$ compared to the corresponding wild-type mice.

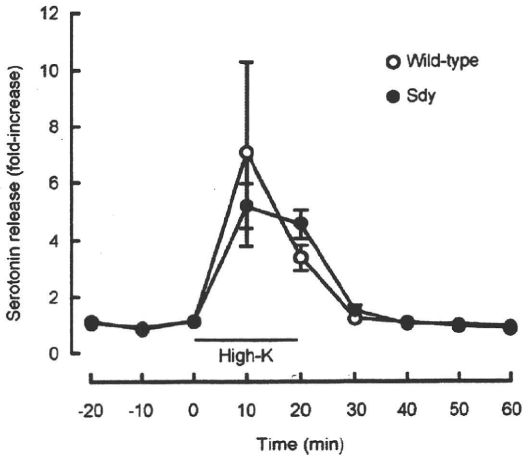

Fig. 3. Depolarization-evoked 5-HT release in the PFC of sdy mice measured by *in vivo* microdialysis. A dialysis probe was inserted through the guide cannula, and perfused with an aCSF at a flow rate of 1.0 μl/min. Following the collection of three baseline fractions, high potassium-containing aCSF (60 mM KCl) was perfused for 20 min through the dialysis probe. Values represent the mean ± S.E. (wild-type, $n = 6$; sdy, $n = 7$).

magnitude of METH-induced hyperlocomotion between wild-type and sdy mice at any doses examined [a two-way ANOVA, genotype, $F_{(1,42)} = 0.346$, $p > 0.05$; genotype by METH treatment interaction, $F_{(2,42)} = 0.046$, $p > 0.05$, Fig. 2A]. In wild-type mice, the locomotor-stimulating effect of METH was potentiated by repeated METH treatment (1.0 mg/kg/day, s.c.) for 5 days [an ANOVA with repeated measures: day after the METH treatment, $F_{(4,56)} = 4.346$, $p < 0.01$, Fig. 2B]. When the time course of METH-induced locomotor sensitization in sdy mice was compared with that in wild-type mice, the sensitization was found to be significantly decreased in sdy mice [an ANOVA with repeated measures: genotype, $F_{(1,14)} = 5.984$, $p < 0.05$; genotype by day after the METH treatment interaction, $F_{(4,56)} = 4.540$, $p < 0.01$, Fig. 2B].

We also investigated the role of dysbindin on the KCl-evoked 5-HT release in the PFC of mice (Fig. 3). Extracellular 5-HT levels in the PFC of wild-type mice were increased by 60 mM KCl stimulation. The KCl-evoked 5-HT release did not differ between wild-type and sdy mice [an ANOVA with repeated measures, genotype, $F_{(1,11)} = 0.010$, $p > 0.05$; time after the KCl treatment, $F_{(5,55)} = 11.921$, $p < 0.01$; genotype by time after the KCl treatment interaction, $F_{(5,55)} = 0.620$, $p > 0.05$]. Although the basal extracellular 5-HT levels were decreased in the PFC of sdy mice, there was no significant difference between the two groups (wild-type, 0.33 ± 0.11 pg/10 μl/10 min; sdy, 0.12 ± 0.01 pg/10 μl/10 min).

Single nucleotide polymorphisms in *DTNBP1* are associated with a reduced cognitive ability [2,3], lower scores on verbal, performance and fullscale IQ tests [34], and deficits in several tasks of attentional response control and/or working memory in schizophrenia [6]. It has been reported that DA content significantly decreased in the cortex, hippocampus and hypothalamus of sdy mice while its metabolite homovanillic acid does not differ between wild-type and sdy mice [19]. Furthermore, sdy mice have been shown to have larger vesicle size, slower quantal release and fewer release events in single cells of the adrenal gland and the hippocampus [4]. Taken together with our findings that the KCl-induced DA release was significantly decreased in the PFC of sdy mice compared with wild-type mice, it is plausible that dysbindin may have a role in the regulation of activity-dependent DA release in the PFC. Dysbindin also plays a role in DA receptor trafficking as shown by increased surface expression of D2 receptors in rat cortical neurons and human neuroblastoma cells following dysbindin siRNA treatment [12]. Presynaptic DA D2 receptors act as autoreceptors, which allow an inhibitory feedback mechanism by altering DA synthesis, release, and reuptake in response to increased synaptic DA. Accordingly, it is possible that decreased levels of extracellular DA in sdy mice may be due to the facilitation of negative feedback through DA D2 receptors in the presynaptic terminals.

Regarding the basal level of extracellular DA, there was no difference between two genotypes in our hands. However, it should be note that no-net flux method is preferred to determine a precise basal level of extracellular DA [23]. Therefore, further study may be required to clarify the role of dysbindin in basal DA release *in vivo*.

The DAergic neuronal activity in the mesolimbic system may be differently affected from that in the mesocortical DAergic system in schizophrenia. Accordingly, we are going to compare the changes in KCl-evoked DA release between the PFC and nucleus accumbens of sdy mice in future.

METH is substrate for DA transporter and competitively inhibits DA uptake and releases DA through reverse transport [27]. METH-induced elevation in extracellular DA leads to hyperlocomotion [27]. The DA efflux through DA transporter requires an increase in Ca^{2+}, and METH increase Ca^{2+} via release of Ca^{2+} from intracellular stores in a manner that is independent of changes in membrane depolarization [9]. Since there was no difference in single METH-induced hyperlocomotion between wild-type and sdy mice, it is unlikely that dysbindin plays a role in METH-induced DA release and hyperlocomotion.

In the present study, we found that repeated METH-induced behavioral sensitization, an animal model of METH psychosis, was significantly attenuated in sdy mice. Repeated treatment with amphetamine produces a persistent increase in the length of dendrites and the number of branched spines on medium spiny neurons [26]. An increase in mRNA levels of the synaptic proteins such as synaptotagmin IV and 25 kDa-synaptic-associated protein has been reported in the cortex, nucleus accumbens, striatum and hippocampus of rats after the repeated treatment with METH [14]. Interestingly, it has been demonstrated that dysbindin gene is associated with METH psychosis [15]. Thus, dysbindin may play a role in repeated METH-induced synaptic changes and DA release, which may underlie the sensitization of the locomotor-stimulating effect of the psychostimulant. Since activity-dependent synaptic plasticity and remodeling of the DAergic system play a crucial role in the development of repeated psychostimulant-induced behavioral sensitization [21], a role of dysbindin in such events should be elucidated in further research.

In contrast to DA release, KCl-induced 5-HT in the PFC of sdy mice did not differ from the release in wild-type mice, although a small reduction of basal extracellular 5-HT levels was observed. Accordingly, it is unlikely that dysbindin regulates the release of all neurotransmitters in the brain. It has been shown that knockdown of dysbindin results in a reduction of stimulus-induced glutamate release in primary cultured cortical neurons, suggesting that dysbindin may regulate release of glutamate [22]. Although it is unclear as to whether the specificity lies with DA and glutamate, it may be determined by the expression levels of as well as target proteins of dysbindin in the brain. A previous study demonstrated high levels of dysbindin mRNA in the substantia nigra and hippocampus in which DAergic and glutamatergic neurons, respectively, exist abundantly [33]. Furthermore, dysbindin is located on the synaptic vesicles, postsynaptic densities, and microtubules of apparent glutamatergic neurons in the hippocampus [32]. To our knowledge, there are no reports indicating the expression of dysbindin mRNA or protein in serotonergic neurons. This possibility should be the subject of further research.

Although the molecular mechanism by which dysbindin regulates depolarization-evoked DA release remains to be determined,

there are several possible explanations. It has been demonstrated that dysbindin binds to snapin [31], SNARE-associated protein implicated in neurotransmission [13]. Recent proteomic analysis has demonstrated that dysbindin directly interacts with Munc18-1 and AP3 complex [11]. Munc18-1 is a neuron specific protein that is essential for the exocytosis of synaptic vesicles [29]. In primary cultured hippocampal neurons, the disbindin-Munc18-1 protein complex is colocalized with synaptophysin [11]. Further studies including electophysiological analysis should be performed to verify the hypothesis that dysbindin may modulate depolarization-evoked DA release through the processes of exocytosis of synaptic vesicles.

Interestingly, disbindin has also been identified as a stable component of biogenesis of lysosome-related organelles complex 1 (BLOC-1) in the brain. The protein level of dysbindin is developmentally regulated, and the dysbindin-containing complex, BLOC-1, plays an important role in the neurite outgrowth [8]. Accordingly, it is possible that the developmental abnormality of DAergic neurons may lead to impaired depolarization-evoked DA release as within the developmental hypothesis of schizophrenia pathogenesis [24].

In conclusion, we demonstrated that depolarization-evoked DA release was diminished in the PFC of sdy mice, whereas KCl-evoked 5-HT release was unaffected. Repeated METH treatment led to the development of behavioral sensitization in wild-type mice, but not in sdy mice. Our findings suggest that dysbindin may have a role in the regulation of depolarization-evoked DA release in the PFC and in the development of behavioral sensitization induced by repeated METH treatment.

Conflict of interest

The authors declare that there are no conflicts of interest in the publication of the present work.

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ORIGINAL INVESTIGATION

Therapeutic potential of nicotine for methamphetamine-induced impairment of sensorimotor gating: involvement of pallidotegmental neurons

Hiroyuki Mizoguchi · Sawako Arai · Hiroyuki Koike · Daisuke Ibi · Hiroyuki Kamei · Toshitaka Nabeshima · Hyoung-Chun Kim · Kazuhiro Takuma · Kiyofumi Yamada

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Abstract
Introduction We have previously found that a disruption to prepulse inhibiton (PPI) induced by methamphetamine (METH) is associated with impaired functioning of pallidotegmental neurons, which play a crucial role in PPI of the startle reflex, through the activation of gamma-aminobutyric acid type B receptors in pedunculopontine tegmental neurons in mice.
Objectives Here, we examined the effect of nicotine on METH-induced impairment of PPI of the startle reflex focusing on dysfunctional pallidotegmental neurons and the neural system.
Results Nicotine (0.15–0.5 mg/kg) ameliorated the deficit in PPI induced by acute METH, and the ameliorating effect of nicotine was antagonized by nicotinic receptor antagonists such as methyllycaconitine and dihydro-β-erythroidine. The acute METH-induced disruption of PPI was accompanied by suppression of c-Fos expression in the lateral globus pallidus (LGP) as well as its induction in the caudal pontine reticular nucleus (PnC) in mice subjected to the PPI test. Nicotine-induced amelioration of PPI deficits in METH-treated mice was accompanied by a reversal of the changes in c-Fos expression in both the LGP and PnC to the basal level.
Conclusions Nicotine is effective in ameliorating the impairment of PPI caused by METH, which may be associated with normalization of the pallidotegmental neurons.

H. Mizoguchi · S. Arai · H. Koike · D. Ibi · K. Takuma · K. Yamada
Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University,
Kanazawa 920-1192, Japan

H. Mizoguchi
Futuristic Environmental Simulation Center,
Research Institute of Environmental Medicine,
Nagoya University,
Nagoya 464-8601, Japan

D. Ibi · K. Yamada (✉)
Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine,
Showa-ku,
Nagoya 466-8560, Japan
e-mail: kyamada@med.nagoya-u.ac.jp

H. Kamei
Laboratory of Clinical Pharmacy Practice and Health Care Management, Faculty of Pharmacy,
Graduate School of Pharmaceutical Sciences, Meijo University,
Nagoya 468-8503, Japan

T. Nabeshima
Department of Chemical Pharmacology,
Graduate School of Pharmaceutical Sciences, Meijo University,
Nagoya 466-8503, Japan

H.-C. Kim
Neuropsychopharmacology and Toxicology Program,
College of Pharmacy, Kangwon National University,
Chuncheon 200-701, South Korea

K. Takuma
Laboratory of Medicinal Pharmacology,
Graduate School of Pharmaceutical Sciences, Osaka University,
Osaka 565-0871, Japan

K. Yamada
CREST-JST,
Nagoya 466-8560, Japan

Keywords Nicotine · Methamphetamine · Schizophrenia · Prepulse inhibition · Memory

Abbreviations

Ach	Acetylcholine
ADHD	Attention deficit hyperactivity disorders
DHβE	Dihydro-β-erythroidine
DRN	Dorsal raphe nucleus
GABA	gamma-aminobutyric acid
LGP	Lateral globus pallidus
METH	Methamphetamine
nAChRs	Nicotinic acetylcholine receptors
PFA	Paraformaldehyde
PnC	Caudal pontine reticular nucleus
PPI	Prepulse inhibiton
PPTg	Pedunculopontine tegmental neurons

Introduction

Prepulse inhibition (PPI) of the startle reflex is viewed as a measure of a process called "sensorimotor gating." Deficits in PPI are observed in patients suffering from certain psychiatric disorders such as schizophrenia (Swerdlow et al. 1994; Castellanos et al. 1996). Over the past 20 years, several studies have shown a primary neural map of brain substrates that control PPI in rodents and humans. Many papers have shown that there are two distinct but interacting neuronal circuits in PPI of the startle reflex, PPI-mediating, and regulating circuits (Fendt et al. 2001; Swerdlow et al. 2001). The acoustic startle response is mediated by the primary mammalian acoustic startle circuit that includes serial connections linking the auditory nerve, the cochlear root neurons, the caudal pontine reticular nucleus (PnC), and the spinal motor neuron (Hoffman and Searle 1968; Fendt et al. 2001; Swerdlow et al. 2001). It is well known that PnC is a critical part of the fast auditory pathway (Fendt et al. 2001; Swerdlow et al. 2001). The PPI-regulating circuit is also the serial connection composed of the hippocampus, prefrontal cortex, and nucleus accumbens (Swerdlow et al. 2001), and recent studies indicate a significant role of the ventral hippocampus–medial prefrontal cortex circuit in PPI (Shoemaker et al. 2005). Additionally, prepulse effects on the startle response may be mediated via the pedunculopontine tegmental neurons (PPTg), which are regulated by descending serial and parallel projections from the forebrain (Swerdlow et al. 2001).

Knowledge of the PPI-regulatory circuit will be an important step towards understanding the neural basis of some of the fundamental underpinnings of cognition (Swerdlow et al. 2001). Despite these studies previously mentioned, however, the neuronal mechanisms and circuits that are involved in PPI of the startle reflex have not yet been completely elucidated (Fendt et al. 2001). We have previously demonstrated that neural circuits containing pallidotegmental gamma-aminobutyric acid (GABA) neurons from the lateral globus pallidus (LGP) to PPTg, play a crucial role in PPI of the acoustic startle reflex in mice (Takahashi et al. 2007). Furthermore, single and repeated treatment with methamphetamine (METH) or MK-801 dose-dependently impaired PPI of the startle reflex (Arai et al. 2008). c-Fos immunohistochemistry revealed that the LGP was activated while the PnC was not after the PPI test in saline-treated control mice. In contrast, the LGP was not activated, whereas the PnC was in the PPI-disrupted mice treated with METH or MK-801. These results suggest that impaired functioning of the pallidotegmental neurons is involved in the disruption of PPI caused by METH and MK-801 in mice (Arai et al. 2008).

Recent studies have presented evidence that nicotine administration or cigarette smoking transiently corrects or ameliorates some of the psychophysiological abnormalities such as impairment of auditory sensory gating found in schizophrenic patients (Adler et al. 1992, 1993). Nicotine has been reported to normalize deficient auditory sensory gating in both schizophrenics and their family members (Adler et al. 1992), and nicotine blocks apomorphine-induced disruption of PPI of the acoustic startle via α7 nAchRs in rats (Suemaru et al. 2004). These studies have led researchers to hypothesize that nicotine has therapeutic effects on the attentional abnormalities in schizophrenia (Adler et al. 1992). However, it remains to be determined how nicotine ameliorates deficits in auditory sensory gating and cognitive dysfunction in schizophrenia.

In the present study, we investigated the effect of nicotine on deficits in PPI in METH-treated mice focusing on dysfunctional pallidotegmental neurons and the neural system.

Materials and methods

Animals

Male ICR mice (Nihon SLC Co., Shizuoka, Japan), 7 weeks old at the beginning of the experiments, were used. The animals were housed in plastic cages (5–6 mice per cage) and kept in a regulated environment (23±1°C, 50±5% humidity) with a 12-h light–dark cycle (lights on at 9:00 h). Food (Labo MR Stock, Nihon Nosan Kogyou Inc., Kanagawa, Japan) and tap water were available ad libitum. The animals were used for the experiments after 7 days of acclimatization to laboratory conditions. All behavioral experiments were carried out between 10:00 and 17:00 h. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of

Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Drugs

METH hydrochloride (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), nicotine (Sigma-Aldrich, St. Louis, MO, USA), methyllycaconitine (Sigma-Aldrich), and dihydro-β-erythroidine (DHβE, Sigma-Aldrich) were dissolved in physiological saline and administered at a volume of 0.1 ml/10 g body weight. The doses examined in the present study were calculated as salt weights.

PPI test

A standard startle box applicable to mice and rats (San Diego Instruments, San Diego, CA) was used. The startle chamber consisted of a Plexiglas tube for mice (105 mm, 38 mm ID, 50 mm OD) placed in a sound-attenuated external box in which animals were individually placed. The tube was mounted on a plastic frame under which a piezoelectric accelerometer was mounted, which recorded and transduced the motion of the tube. Animals were randomly divided into nonstimulated and PPI groups and subjected to the behavioral test only once. Mice were placed into the chamber in the PPI test cage and then habituated to the experimental environment for 10 min with 65 dB of background white noise. The PPI test has three continuous sessions: (1) five startle trials; (2) ten startle trials, ten no-stimulus trials, and 40 PPI trials; and (3) five startle trials. The intertrial interval was between 10 and 20 s, and the total duration of the three sessions was 17 min. The startle trial consisted of a single 120-dB white noise burst lasting 40 ms. The PPI trials consisted of a prepulse (20 ms burst of white noise with any intensity of 69, 73, 77, or 81 dB) followed, 100 ms later, by the startle stimulus (120 dB, 40-ms white noise). Each of the four prepulse trials (69, 73, 77, or 81 dB) was presented ten times. During the no-stimulus trial, no stimulus was presented but the movement of the animal was scored. Sixty different trials were presented pseudorandomly, ensuring that each trial was presented ten times and that no two consecutive trials were identical. The resulting movement of the animal in the external box was measured during 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the ten startle trials. PPI was calculated according to the formula: $100 \times [1 - (PPx/P120)]\%$ in which PPx was the mean of the ten PPI trials (PP69, PP73, PP75, or PP80), and P120 was the basal startle amplitude (Ellenbroek et al. 2002; Takahashi et al. 2007).

Nonexposed control mice were placed into the chamber in the PPI test cage and habituated to the experimental environment for 10 min with 65 dB of background white nose as was the experimental group. They were kept in the chamber for any additional 17 min without any startle or PPI trials.

Mice were given saline or METH (3 mg/kg, sc) 15 min before the PPI test. Nicotine (0.1 or 0.5 mg/kg, sc) was administered 15 min before the saline or METH. Methyllycaconitine, a selective α7 subunit-containing nAChR (α7 nAChR) antagonist, and DHβE, a α4β2 subunit-containing nAChR (α4β2 nAChR) antagonist, were given 15 min before nicotine treatment.

Fos immunohistochemistry

c-Fos immunostaining was performed as described previously (Takahashi et al. 2007; Arai et al. 2008). As Fos expression was shown to occur from 1 to 4 h after a single short stimulation, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg) 2 h after the PPI test. Alternatively, mice were treated with saline or METH (3 mg/kg, sc) and killed 2.5 h after treatment without the PPI test. The animals were transcardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brains were removed, post-fixed in the same fixative for 2 h, and then cryoprotected in 30% sucrose in PBS. Frozen serial coronal sections (20 mm) of the entire brain were made and incubated with 10% goat serum and 0.1% Triton X-100 in 0.1-M phosphate buffer and then incubated with rabbit anti-c-Fos antibody (1:200; sc-253, Santa Cruz Biotechnology, CA) for 24 h at 41°C. They were washed with phosphate buffer and incubated with biotinylated goat anti-rabbit antibody (1:200; BA-1000, Vector Laboratories, Burlingame) at room temperature for 1 h. The sections were washed and processed with avidin-biotinylated horseradish peroxidase complex (Vector ABC kit, Vector Laboratories) and the reaction was visualized using diaminobenzidine.

Quantitative analysis of c-Fos immunohistochemistry

To quantify the number of Fos-positive cells in the brain, we used a fluorescence microscope with a cooled CDD digital camera system (Axio Imager A1/AxioCam MRc5; Carl Zeiss, Jena, Germany) to scan the sections and calculated the cell numbers from the digitized images using image-analyzing software Win ROOF (ver. 5.6, Mitani Co., Fukui, Japan). After analyzing effects of various drugs treatments on PPI, four or five mice in each treatment group that showed typical PPI responses (the values startle amplitude and PPI at 69–81 dB were

within the range of mean ±SE) were used for c-Fos experiment. We selected three different sections from each animal and defined the region of interest (ROI), 500×500 pixels (168×168 μm), using the software in both the right and left hemispheres of the sections according to a mouse brain atlas (Franklin and Paxinos 1997). To avoid double counting errors, we chose a counting protocol in the software, which does not calculate cell numbers at the border of a ROI. The counting of Fos-positive cells was repeated three times with differential ROI in one section. This procedure resulted in a total of nine determinations of the number of Fos-positive cells within a specified area for each brain. The average of the resulting nine determinations of the c-Fos-positive cell numbers was used as a measure of each animal for statistical analysis (Takahashi et al. 2007; Arai et al. 2008). c-Fos-positive cells were counted by an individual blind to the treatment conditions. In the drug treatment test, the areas selected were LGP and PnC.

Statistical analysis

All data were expressed as the mean ±SE. Statistical significance was determined by Student's *t* test for two group comparisons and one-way analysis of variance (ANOVA) for multigroup comparisons in experiments counting c-Fos-positive cell counts and by a repeated measure ANOVA for PPI response measurements. Student Newman–Keuls test was used for post hoc comparisons when *F* values were significant ($p<0.05$).

Results

Effect of nicotine on METH-induced impairment of PPI

METH at a dose of 3 mg/kg significantly reduced PPI compared with saline-treated control group (Fig. 1b; $F(1,32)=31.2$, $p<0.0001$). METH at a 3 mg/kg increased startle amplitude (160% of control), but the effect was not statistically significant (Fig. 1a; $p=0.0713$ by *t* test). Nicotine (0.15–0.5 mg/kg, sc) had little effect on startle amplitude in saline-treated control group (Fig. 1a; $F(2,30)=1.60$, $p=0.2197$) or METH-treated group (Fig. 1a, $F(2,32)=3.30$, $p=0.0497$), but it reversed the impairment of PPI induced by METH. For the influence of nicotine on METH-induced deficits in PPI, there were significant effects of drug treatment (Fig. 1b, $F(5,62)=14.9$, $p<0.0001$ by repeated one-way ANOVA), prepulse intensity ($F(3,186)=59.2$, $p<0.0001$ by repeated one-way ANOVA), and their interaction ($F(15,186)=2.26$, $p=0.0059$ by repeated one-way ANOVA).

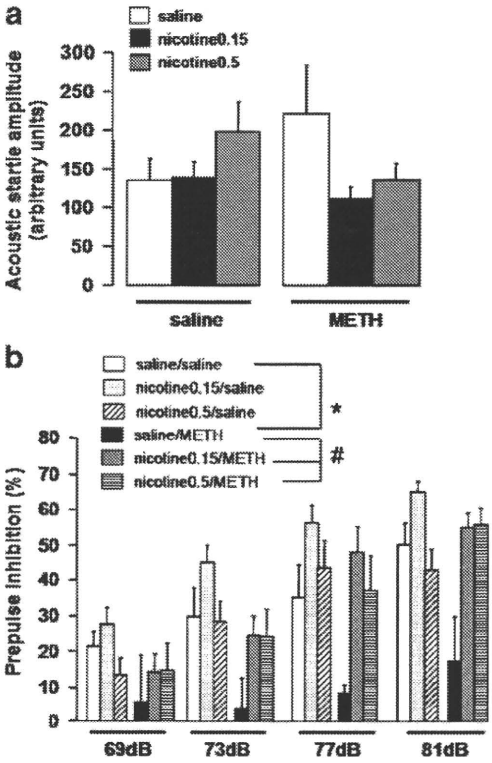

Fig. 1 Effect of nicotine on acute METH-induced impairment of PPI in mice. Mice were treated with nicotine (0.15–0.5 mg/kg, sc) 15 min before receiving METH (3 mg/kg, sc). **a** Acoustic startle amplitude as measured in trials without a prepulse. **b** PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). Values are the mean±SE (saline/saline, $n=18$; saline/METH: $n=16$; nicotine 0.15/saline, $n=9$; nicotine 0.15/METH, $n=11$; nicotine 0.5/saline, $n=6$; nicotine 0.5/METH, $n=8$). *$p<0.05$ vs. saline/saline. #$p<0.05$ vs. saline/METH

Effect of nicotinic receptor antagonists on recovery of METH-induced deficits in PPI by nicotine

Firstly, we examined the effects of methyllycaconitine, an α7 nAchR antagonist, and DHβE, an α4β2 nAchR antagonist, alone on PPI of the startle reflex in mice. Neither antagonists at doses of 1–2 mg/kg had significant effects on the startle amplitude (Fig. 2a; methyllycaconitine, $F(2,22)=0.21$, $p=0.8092$; DHβE, $F(2,19)=1.39$, $p=0.2724$) or PPI (Fig. 2b; methyllycaconitine, drug treatment, $F(2,22)=0.33$, $p=0.7245$; prepulse intensity, $F(3,66)=21.0$, $p<0.0001$; interaction, $F(6,66)=1.39$, $p=0.2331$; DHβE, drug treatment, $F(2,19)=0.22$, $p=0.8020$; prepulse intensity, $F(3,57)=17.4$, $p<0.0001$; interaction, $F(6,57)=0.12$, $p=0.9940$).

Next, we examined the effect of nicotinic receptor antagonists on recovery of METH-induced deficits in PPI by nicotine (0.15 mg/kg). There was no difference in the startle amplitude between saline and METH-treated group (Fig. 3, $p=0.3377$ by *t* test). METH at a dose of 3 mg/kg significantly reduced PPI compared with saline-treated control group as well as the result in Fig. 1b (Fig. 3, $F(1,29)=20.6$, $p=0.0001$). Prior treatment with methylly-

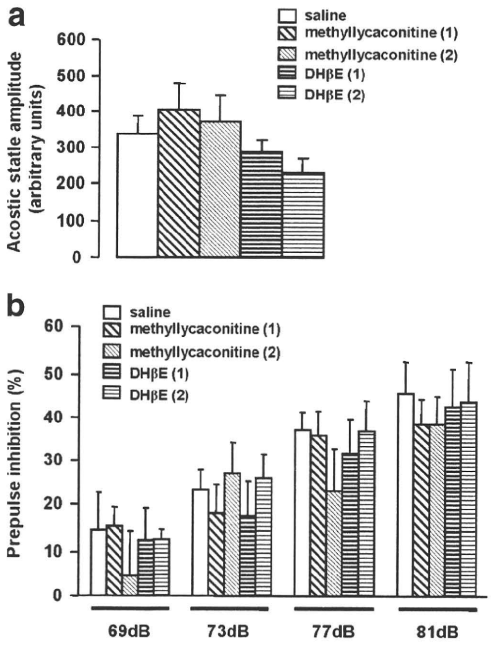

Fig. 2 Effects of a selective α7 subunit-containing nAChR antagonist, methyllycaconitine, and α4β2 subunit-containing nAChR antagonist, DHβE, on acoustic startle amplitude (**a**) and PPI (**b**) in mice. Mice were treated with methyllycaconitine (1-2 mg/kg, sc) or DHβE (1–2 mg/kg) 45 min before the PPI test. Values are the mean±SE (A, saline, $n=7$; methyllycaconitine 1 mg/kg, $n=8$; methyllycaconitine 2 mg/kg, $n=10$; DHβE 1 mg/kg, $n=8$; DHβE 2 mg/kg, $n=7$)

caconitine significantly increased the startle amplitude in METH with nicotine group (Fig. 3a, $F(3,45)=4.01$, $p=0.013$ by one-way ANOVA) and dose-dependently blocked the reversal of the METH-induced disruption to PPI by nicotine (Fig. 3a, drug treatment, $F(3,45)=7.71$, $p=0.0003$; prepulse intensity, $F(3,135)=63.3$, $p<0.0001$; interaction, $F(9,135)=1.45$, $p=0.1750$).

Likewise, the reversal of METH-induced disruption to PPI by nicotine was dose-dependently eliminated by DHβE (Fig. 3b, drug treatment, $F(3,46)=9.08$, $p=0.0001$; prepulse intensity, $F(3,138)=60.6$, $p<0.0001$; interaction, $F(9,138)=1.88$, $p=0.0598$) without any effects on startle amplitude in METH with nicotine group ($F(3,46)=2.62$, $p=0.0619$ by one-way ANOVA).

Effects of nicotine on the METH-induced changes in c-Fos expression in the LGP and PnC of mice subjected to the PPI test

To study the effects of drug treatment on pallidotegmental neural activation after the PPI test, we examined c-Fos expression immunohistochemically in the LGP and PnC of mice treated with saline and METH (3 mg/kg, sc). Representative photomicrographs of c-Fos staining in the LGP and PnC after the PPI test are shown in Figs. 4 and 5, respectively. Quantitative analysis of the staining indicated a significant difference in the number of c-Fos-positive cells in the LGP (Fig. 4, $F(4,16)=44.8$, $p<0.0001$ by one-way ANOVA). There was a significant increase in the number of c-Fos-positive cells in saline-treated mice subjected to the PPI test ($n=4$) compared with nonexposed control mice ($n=5$; $p=0.0001$). The number of c-Fos-positive cells in the LGP of mice treated with METH ($n=4$) was significantly less than that in saline-treated control mice subjected to the PPI test (Fig. 4, $p<0.0001$).

To study the effects of nicotine (0.15 mg/kg, sc) on METH-induced changes in neural activity associated with the PPI deficits, we examined c-Fos expression immunohistochemically in the LGP of mice after the PPI test. Pretreatment with nicotine significantly increased the number of c-Fos-positive cells in the LGP compared with the METH-treated group ($n=4$; $p<0.0001$), although nicotine had no effect in saline-treated control mice (Fig. 4).

A significant change in c-Fos expression was also observed in the PnC (Fig. 5, $F(4,15)=11.7$, $p=0.0002$ by one-way ANOVA). There was no difference in the number of c-Fos-positive cells between nonexposed control mice ($n=4$) and saline-treated mice subjected to the PPI test ($n=4$). The number of c-Fos-positive cells in the PnC of mice treated with METH ($n=4$) was significantly increased compared with that in saline-treated mice ($p=0.0003$). Although nicotine itself had no effect on c-Fos expression in the PnC, the METH-induced increase in the number of c-Fos-positive cells in PnC was suppressed by pretreatment with nicotine (Fig. 5, $F(4,15)=11.7$, $p=0.0002$ by one-way ANOVA).

Discussion

Deficits of PPI in psychosis and schizophrenia can be mimicked in rodents by treatment with psychostimulants such as METH (Arai et al. 2008; Dai et al. 2004). Acute METH-induced PPI impairment in mice was accompanied by an increase in startle amplitude. Amphetamine is also reported to increase startle response (Davis 1988). Thus, the alterations of startle response may account, at least in part, for the METH-induced disruption of PPI. Our findings showed that nicotine ameliorated the disruption to PPI induced by METH via both α7 and α4β2 nicotinic receptors (Figs. 1, 2 and 3). Nicotine blocked a dopamine agonist-induced disruption of PPI of the acoustic startle response via central α7 nicotinic receptors in rats (Suemaru et al. 2004). A α7 nicotinic receptor agonist reversed PPI deficits in isolation-reared rats suggesting the activation of α7 nicotinic receptors to be of benefit in reestablishing an efficient gating function (Cilia et al. 2002). Stimulation of heteromeric nicotine acetylcholine receptors containing both α and β subunits, and possibly of the α4/β2 type, affects

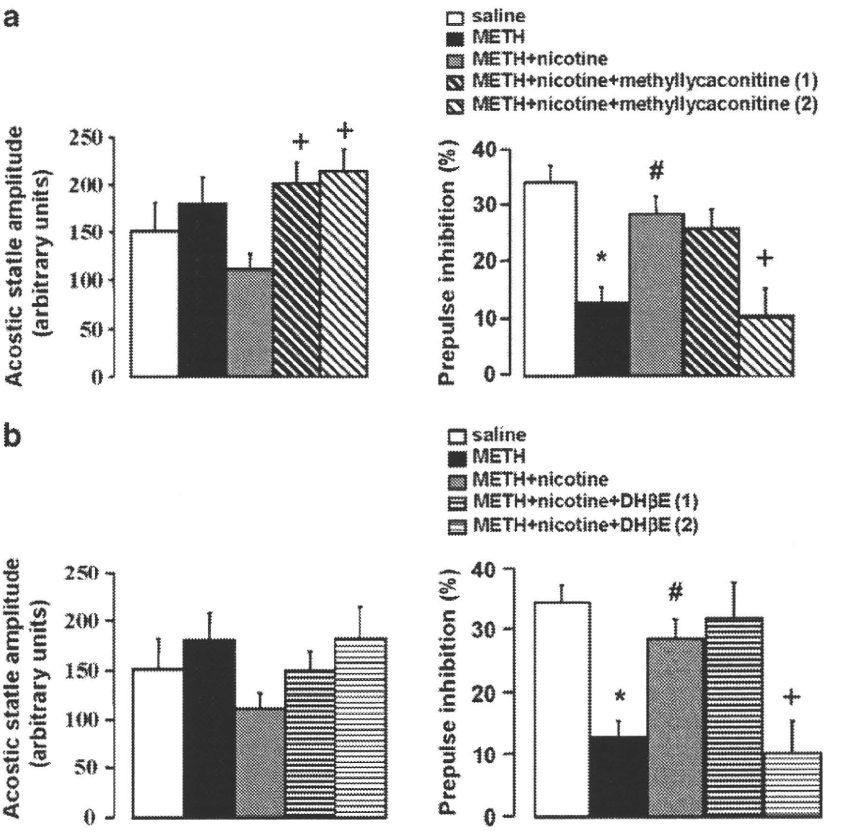

Fig. 3 Effects of a selective α7 subunit-containing nAChR antagonist, methyllycaconitine, (**a**) and α4β2 subunit-containing nAChR antagonist, DHβE, (**b**) on the ameliorating effects of a nicotine agonist on the METH-induced PPI deficit in mice. Mice were treated with methyllycaconitine (1–2 mg/kg, sc) or DHβE (1–2 mg/kg) 15 min before receiving nicotine (0.15 mg/kg, sc). Results of PPI test are shown as average of various PPI test from 69–81 dB. Values are the mean±SE (A, saline, $n=14$; METH, $n=17$; METH with nicotine, $n=16$; METH with nicotine and methyllycaconitine 1 mg/kg, $n=7$; METH with nicotine and methyllycaconitine 2 mg/kg, $n=9$; B, saline, $n=14$; METH, $n=17$; METH with nicotine, $n=16$; METH with nicotine and DHβE 1 mg/kg, $n=9$; METH with nicotine and DHβE 2 mg/kg, $n=8$). *$p<0.05$ vs. saline-treated control group. #$p<0.05$ vs. METH-treated group. +$p<0.05$ vs. METH with nicotine group

sensorimotor gating (Schreiber et al. 2002). Our findings are consistent with these previous reports and support that nicotine has a beneficial effect on PPI deficits. Alternatively, α6-containing nicotinic receptors may play a role since both methyllycaconitine (Klink et al. 2001) and DHβE (Exley et al. 2008) have high affinity for these receptors.

As shown in Fig. 1, nicotine at a dose of 0.15 mg/kg treatment apparently increased PPI in saline-treated mice although such potentiation by nicotine was not observed at a higher dose (0.5 mg/kg). It is possible that nicotine may have biphasic effects on PPI. In fact, previous studies have shown that nicotine increases (Acri et al. 1995) or decreases (Schreiber et al. 2002) sensorimotor gating by disparities in the dose, prepulse intensity, age, species, and strain. In contrast, our data showed that both low and high doses of nicotine ameliorated PPI impairment in METH-treated mice. Thus, the effect of nicotine on PPI may depend on the subject's condition (saline or METH-treated mice).

We have previously demonstrated that GABAergic neurons in the LGP, which project directly to the PPTg, are activated by a prepulse stimulus regardless of startle pulse stimuli, and that the pallidotegmental GABAergic neurons act as an interface between the brainstem PPI-mediating and the forebrain PPI-regulating circuits (Takahashi et al. 2007). In the present study, we demonstrated that the disruption of PPI caused by METH was accompanied by impairment of the LGP and hyperactivation of the PnC, which manifested as changes of c-Fos expression in the LGP and PnC after the PPI test, and it is reasonable to assume that METH disrupts PPI of the startle reflex in mice by inhibiting the activation of pallidotegmental GABAergic neurons evoked by a prepulse stimulus (Arai et al. 2008). Previously, as a control experiment, we measured the effects of METH (3 mg/kg, sc) on c-Fos expression in the LGP and PnC of mice that were not subjected to the PPI test. Single METH treatment had no effect on c-Fos expression in the LGP and PnC (Arai et al. 2008, supplementary Fig. 1). From this control experiment, it is obvious that the effects of METH on c-Fos expression in mice that were subjected to the PPI test are markedly different from those found in mice that were not subjected to the test (Arai et al. 2008). In this study, the ameliorating effect of nicotine on PPI was associated with the normalization of PnC hyperactivation, and nicotine increased the number of c-Fos-positive cells in the LGP of METH-treated mice subjected to the PPI test. Thus, nicotine may modulate pallidotegmental GABAergic neurons resulting in inhibition of the activation in the PnC induced by METH. However, it is clear that further studies are required to clarify the neurobiological mechanisms underlying the deficit in PPI induced by METH as well as the ameliorating effect of nicotine on the disruption.