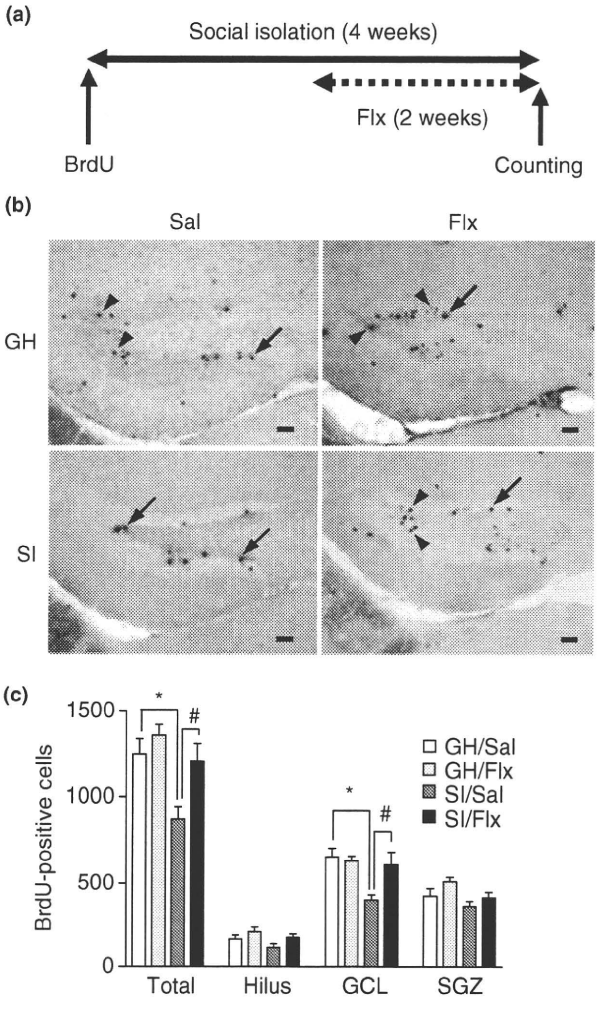

Fig. 7 Effect of repeated fluoxetine (Flx) treatment on social isolation (SI)-induced impairment of the survival of newly divided cells in the dentate gyrus of hippocampus. 5-bromo-2′-deoxyuridine (BrdU; 75 mg/kg, i.p.) was injected three times at 2 h intervals one day before starting 4-week isolation. Daily administration of Flx (10 mg/kg, i.p.) was started 2 weeks after SI and continued for 2 weeks. Animals were killed after SI, and BrdU-positive cells in the subgranular zone (SGZ), hilus, and granule cell layer (GCL) were counted as described in *Materials and methods*. GH/Sal: saline-treated group-housed mice ($n = 6$), GH/Flx: fluoxetine-treated GH mice ($n = 6$), SI/Sal: saline-treated SI mice ($n = 7$), SI/Flx: fluoxetine-treated SI mice ($n = 7$). (a) Experimental schedule. (b) Representative photographs showing the distribution of BrdU-positive cells in GH/Sal, GH/Flx, SI/Sal, and SI/Flx mice, respectively. Scale bar: 200 μm. (c) Total numbers of BrdU-positive cells were expressed as the sum of the number in the SGZ (arrows), hilus, and GCL (arrowheads). Values indicate the mean ± SE. $^{*}p < 0.05$ versus GH/Sal, $^{\#}p < 0.05$ versus SI/Flx.

repeated Flx treatment ($p < 0.05$). The same result was observed in the number of BrdU-positive cells in the GCL (Fig. 7c). Furthermore, there was a significant difference in the ratio of NeuN-positive cells among BrdU-labeled newly divided cells in the hilus + SGZ + GCL between SI/Sal (69.4 ± 0.6%, $n = 3$) and SI/Flx (78.0 ± 1.1%, $n = 3$; $p < 0.01$) mice.

Changes in gene expression in the DG of hippocampus by SI

To compare the changes in gene expression in the DG of hippocampus between GH and SI mice, we used Affymetrix GeneChip mouse genome assays. Scatter plots and hierarchical clustering analysis showed no obvious changes in global expression profiles between GH and SI groups. In an attempt to detect gene-specific significant changes in expression between GH and SI groups, a general linear model incorporating the feeding period was used. Among genes whose *p*-value of change between GH and SI groups was less than 0.01 and whose absolute value of log2-fold change was larger than 0.263 (1.2-fold), one gene (0.0022%) was increased and 21 (0.047%) genes were decreased in the expression levels in SI mice compared to GH mice (Table 1). Three genes, *Nurr1*, *Npas4*, and a gene of unknown function AK003534 had a false discovery rate less than 0.5. We confirmed by the real-time RT-PCR that the expression levels of *Nurr1* and *Npas4* mRNA in the DG of hippocampus were significantly reduced after 4-week SI compared with those in GH controls (Fig. 8).

Discussion

In the present study, we found that long-term SI after weaning in mice had little effect on the proliferation of newly divided cells in the hippocampus as measured by BrdU labeling. In contrast, Lu *et al.* (2003) previously reported that SI in early life reduces cell proliferation in the DG of rats. Apparently, our present findings are inconsistent with their findings, but the discrepancy may be explained by the difference of the administration schedule of BrdU and/or species difference used. We administrated BrdU three times at 2 h intervals and then counted the number of BrdU-labeled cells 24 h after the last BrdU injection, while Lu *et al.* (2003) administrated BrdU twice daily on the last 3 days of the rearing treatment. Furthermore, because neurogenesis is affected by genetic background (Jacobs *et al.* 2000), hereditary differences between rats and mice may result in distinct effects on cell proliferation.

The present study indicates that the survival and differentiation of newly divided cells in the hippocampus are reduced by SI after weaning in mice, which is consistent with previous studies in rats and guinea pigs (Lu *et al.* 2003; Rizzi *et al.* 2007). It is possible that SI may induce the cell death of newly divided cells (Rizzi *et al.* 2007), but we could not detect apoptotic cells labeled by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling in the DG of either GH or SI mice (unpublished observation). Further studies are required to clarify the mechanism by which the survival of newly divided cells was impaired by SI after weaning. The present study also indicates that cell survival of newly divided

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Table 1 List of genes that the expression in the DG of the hippocampus was altered by SI

Gene title	Public ID	Gene symbol	Probe	Expression ratio		
				3 days	2 weeks	4 weeks
Euchromatic histone methyltransferase 1	BB409568	*Ehmt1*	1454776_at	1.12	1.17	1.54
Neuronal PAS domain protein 4	AV348246	*Npas4*	1459372_at	0.74	0.36	0.76
Activity regulated cytoskeletal-associated protein	NM_018790	*Arc*	1418687_at	0.67	0.61	0.76
FBJ osteosarcoma oncogene	AV026617	*Fos*	1423100_at	0.76	0.57	0.75
RIKEN cDNA C330006P03 gene	BB398124	*C330006P03Rik*	1436387_at	0.83	0.60	0.78
Nuclear receptor subfamily 4, group A, member 2 (Nr4a2), mRNA	BB703394	*Nr4a2 (Nurr1)*	1455034_at	0.77	0.67	0.78
Nuclear receptor subfamily 4, group A, member 2	BB322941	*Nr4a2*	1447863_s_at	0.72	0.68	0.85
Vacuolar protein sorting 52 (yeast)	BB429200	*Vps52*	1447894_x_at	0.60	0.89	0.85
Nuclear receptor subfamily 4, group A, member 1	NM_010444	*Nr4a1*	1416505_at	0.82	0.65	0.91
Early growth response 1	NM_007913	*Egr1*	1417065_at	0.78	0.76	0.87
Corticotropin releasing hormone binding protein	AI854101	*Crhbp*	1436127_at	0.87	0.82	0.71
Period homolog 2 (Drosophila)	AF035830	*Per2*	1417602_at	0.75	0.84	0.83
16 days embryo head cDNA, RIKEN full-length enriched library, clone: C130019I03 production: unclassifiable, full insert sequence	BB363968	–	1460098_at	0.88	0.83	0.74
Kinesin family member 1B	BE199508	*Kif1b*	1425270_at	0.77	0.91	0.78
ADP-ribosylation factor 4-like	NM_025404	*Arfl4*	1418250_at	0.81	0.77	0.89
Poliovirus receptor	BB049138	*Pvr*	1451160_s_at	0.90	0.71	0.87
Nuclear receptor subfamily 4, group A, member 2	NM_013613	*Nr4a2*	1450750_a_at	0.77	0.80	0.92
Homer homolog 1 (Drosophila)	AF093257	*Homer1*	1425671_at	0.92	0.68	0.89
Dihydrouridine synthase 4-like (*Saccharomyces cerevisiae*)	AK010138	*Dus4l*	1453252_at	0.73	0.83	0.93
Diacylglycerol kinase, iota	BE647270	*Dgki*	1439986_at	0.90	0.72	0.87
PHD finger protein 17	BG065238	*Phf17*	1452180_at	0.91	0.82	0.74
Dual specificity phosphatase 1	NM_013642	*Dusp1*	1448830_at	1.01	0.67	0.85

SI, social isolation; DG, dentate gyrus.

Mice were killed after 3 days, 2 weeks, and 4 week of SI. Brain sample (hippocampal DG) from four mice were pooled and used for the DNA microarray. Values indicated the mean (n = 5–6, each from four mice).

cells is diminished in the GCL, while neuronal differentiation is only diminished in the SGZ. It is possible that development of stem-like cells in the SGZ is repressed by SI. Some of those cells in the SGZ of SI mice could not grow up to NeuN-positive cells. Therefore, SI may decrease differentiation to NeuN-positive cells but not cell number in the SGZ.

In the present study, the duration of Flx treatment in the behavioral experiment (3 weeks, Figs 5 and 6) was different from that in the experiment of BrdU labeling (2 weeks, Fig. 7). Although we did not examine whether the different duration of treatment with Flx affected the enhancing effect on neurogenesis in the hippocampus or not, a previous study demonstrated that the effect of repeated Flx treatment for 2 weeks on neurogenesis was similar to that for 4 weeks (Malberg *et al.* 2000). Therefore, we suggest that Flx treatment for 3 weeks in the behavioral experiment may have effects on the number of BrdU-positive cells in the SGZ, similar to those observed after the repeated treatment for 2 weeks.

Current study showed that Flx administration completely prevented the SI-induced impairment of survival of newly divided cells and spatial learning and memory, but not aggression completely, because spatial learning and memory are more strongly associated with the hippocampal function than aggression (Davidson *et al.* 2000; Jhoo *et al.* 2004; Miyamoto *et al.* 2005). To demonstrate our thoughts as described above, further experiments are required.

A recent study showed that an appropriate activity of NMDA receptors plays a role in the survival of newly divided cells in the DG of the hippocampus (Tashiro *et al.* 2006). SI in rats induces the expression of the NMDA receptor, NR1A mRNA in the DG of the hippocampus (Hall *et al.* 2002). Furthermore, it is reported that SI stress elevates circulating levels of corticosterone (Stranahan *et al.* 2006) and that a high dose of corticosterone induces glutamate release in the hippocampus (Karst *et al.* 2005; Venero and Borrell 1999;. Thus, long-term SI after weaning may induce glutamate release and the expression of NR1A subunit of NMDA receptor in the hippocampus, leading to aberrant NMDA receptor activation. As NMDA receptor activation is reported to induce the cell death of stem-like cells *in vitro* (Tashiro *et al.* 2006; Asahi *et al.* 1998), it is possible that

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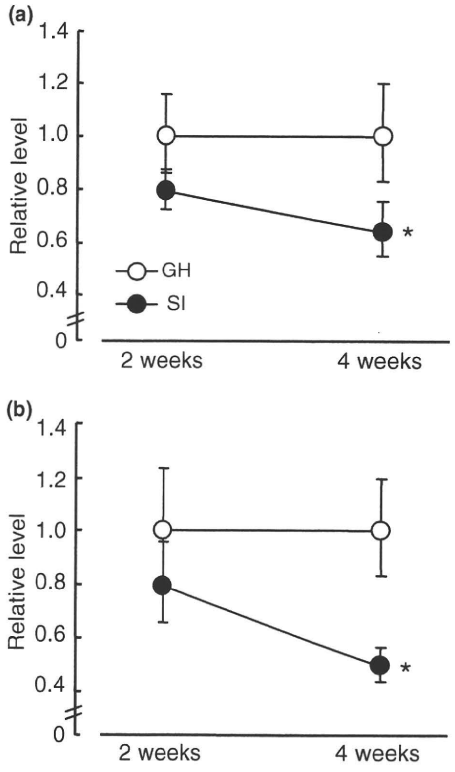

Fig. 8 Changes in the expression of nuclear receptor subfamily 4, group A, member 2 (a) and neuronal PAS domain 4 (b) mRNA in the dentate gyrus of the hippocampus after 2-and 4-week social isolation. Values indicated the mean ± SE (n = 5–10, each from two mice). *p < 0.05 versus group-housed (two-tailed t-test).

SI-induced alternation of NMDA receptor signaling may reduce the survival of newly divided cells in the DG of the hippocampus in SI mice.

It has been demonstrated that GABA plays a crucial role in the differentiation of stem-like cells in the SGZ of the hippocampus and that excitatory GABAergic input to stem-like cells in the hippocampus increases the expression of the transcription factor NeuroD, a positive regulator of neuronal differentiation in the DG (Tozuka *et al.* 2005). A further *in vivo* study using $GABA_A$ receptor agonists indicated the promotion of neural differentiation via GABAergic excitation (Li and Pleasure 2005; Tozuka *et al.* 2005). Pinna *et al.* (2006) showed that SI alters the subunit expression of $GABA_A$ receptor leading to dysfunction of the $GABA_A$ receptor agonist-induced sedative effect. Thus, it is also possible that the dysfunction of $GABA_A$ receptors may be involved in the impairment of differentiation of newly divided cells in the DG of the hippocampus induced by long-term SI after weaning in mice.

It is well known that SI induces impairment of emotion-related behaviors and cognition (Wongwitdecha and Marsden 1996; Weiss *et al.* 2004). Consistently, in the present study, we found that long-term SI in mice after weaning impairs spatial learning and memory and induces impulsiveness and aggressive behavior. These behavioral abnormalities in SI mice resemble the symptoms observed in patients suffering from attention-deficit hyperactivity disorder and depression (Castellanos and Tannock 2002; Heim *et al.* 2004). Notably, memory deficit, impulsiveness/aggressive behavior as well as the impairment of survival of newly divided cells in the hippocampus of SI mice were reversed by repeated Flx administration. A single Flx treatment failed to reverse impulsiveness/aggressive behavior and anxiety in SI mice (data not shown). Our findings are in agreement with the previous study that impairment of cell survival induced by experiences in early life (e.g., maternal separation) was reversed by repeated Flx administration in rats (Lee *et al.* 2001). Although the causal relationship between behavioral deficits and impairment of neurogenesis in SI mice is unclear, a previous study suggested that the behavioral effects of chronic Flx may be mediated by the stimulation of neurogenesis in the hippocampus because X-ray irradiation to a restricted region of the mouse brain containing the hippocampus prevented the neurogenic and behavioral effects of Flx (Santarelli *et al.* 2003).

Regarding the mechanism of action of Flx on SI-induced impairment of neurogenesis, it is reasonable to assume the involvement of 5-hydroxytryptamine (5-HT) system because of the selective inhibitory effect of 5-HT reuptake. Previous studies demonstrated that SI induces impairment of the 5-HT system in the hippocampus (Bickerdike *et al.* 1993; Whitaker-Azmitia *et al.* 2000; Muchimapura *et al.* 2002; Preece *et al.* 2004). For instance, previous studies have shown that 5-HT release is reduced in the hippocampus of isolated rats under aversive conditions and following the administration of parachloroamphetamine (a 5-HT-releasing drug) (Bickerdike *et al.* 1993; Muchimapura *et al.* 2002). Regarding changes in 5-HT receptors in the hippocampus, Preece *et al.* (2004) showed that SI from weaning in rats results in alternations of 5-HT_{1A} and 5-HT_{2A} receptor density in the frontal cortex and hippocampus. Gould (1999) suggested that $5HT_{1A}$ receptors are located on the hippocampal stem-like cells. Accordingly, activation of 5-HT_{1A} receptors is required for the effects of Flx on behavior and neurogenesis (Santarelli *et al.* 2003). Furthermore, because NMDA and $GABA_A$ receptors play a role in the survival and differentiation of newly divided cells in the DG of the hippocampus as described above, these receptors may also be involved in the effect of Flx on neurogenesis (Zhong and Yan 2004; Yuen *et al.* 2005).

In the present study, we demonstrated that SI significantly reduced the mRNA levels of *Nurr1* and *Npas4* in the DG of hippocampus. In agreement with our finding, it is reported that both *Nurr1* and *Npas4* mRNA are highly expressed in the hippocampus (Xiao *et al.* 1996; Moser *et al.* 2004). Neuronal PAS domain 4 (Npas4) has constitutive or developmental functions which may be critical for regulating the

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transcriptional control of limbic patterning and function (Moser *et al.* 2004). Nuclear receptor subfamily 4, group A, member 2 (Nurr1) is essential for both survival and final differentiation of dopaminergic precursor neurons into a complete dopaminergic phenotype (Saucedo-Cardenas *et al.* 1998). Although we did not examine the alteration of *Nurr1* and *Npas4* mRNA expression in the BrdU-positive cells in the hippocampus, it is possible that Nurr1 and Npas4 may contribute to the impairment of neurogenesis and memory, and aggression in SI mice. In the present study, it is unclear that which regions of the DG *Nurr1* and *Npas4* mRNA expression are altered, because the microarray and RT-PCR in present study were carried out without dissecting multiple regions of the DG, and that whether Flx recovers SI-induced alteration of these genes. Further studies are required to test this assumption and attenuate the limitation. These additional studies will be useful to understand the molecular mechanisms underlying SI-induced impairment of hippocampal neurogenesis.

In conclusion, the present study demonstrated that long-term SI in mice after weaning reduced survival and differentiation but not the proliferation of newly divided cells in the DG of the hippocampus. In parallel, long-term SI in juvenile mice induced hippocampal dysfunction which was manifested by the development of learning and memory impairment as well as impulsiveness/aggressive behavior. Furthermore, we demonstrated that SI-induced impairment of neurogenesis, cognition and emotion-related behaviors were reversed by repeated Flx administration. The DNA microarray and real-time RT-PCR analyses indicated that long-term SI after weaning in mice affects the expression of a very few genes (less than 0.1%) in the DG of hippocampus and that the expression of Nurr1 and Npas4 mRNA was significantly reduced by long-term SI.

Thus, our findings suggested that long-term deprivation of communication with others in juveniles impairs the mechanism of neurogenesis in the hippocampus, which could be involved in the development of psychiatric disorders with impairment of emotion-related behaviors and cognition. Furthermore, Flx may be effective in treating impairment of emotion-related behaviors and memory in which poor environmental conditions and/or social interaction in early life could be involved in the pathogenesis.

Disclosure/Conflicts of interest

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

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Involvement of Pallidotegmental Neurons in Methamphetamine- and MK-801-Induced Impairment of Prepulse Inhibition of the Acoustic Startle Reflex in Mice: Reversal by $GABA_B$ Receptor Agonist Baclofen

Sawako Arai[1,7], Kazuhiro Takuma[1,7], Hiroyuki Mizoguchi[1,2], Daisuke Ibi[1], Taku Nagai[3], Kenji Takahashi[1], Hiroyuki Kamei[4], Toshitaka Nabeshima[5] and Kiyofumi Yamada*[,1,3,6]

[1]Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan; [2]Futuristic Environmental Simulation Center, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan; [3]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan; [4]Laboratory of Clinical Pharmacy Practice and Health Care Management, Meijo University, Nagoya, Japan; [5]Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan; [6]CREST, JST, Nagoya, Japan

We have previously demonstrated that pallidotegmental GABAergic neurons play a crucial role in prepulse inhibition (PPI) of the startle reflex in mice through the activation of $GABA_B$ receptors in pedunculopontine tegmental neurons. In this study, we investigated whether PPI disruption induced by methamphetamine (METH) or MK-801 is associated with the dysfunction of pallidotegmental neurons. Furthermore, we examined the effects of baclofen, a $GABA_B$ receptor agonist, on METH- and MK-801-induced PPI impairment. Acute treatment with METH (3 mg/kg, subcutaneouly (s.c.)) and MK-801 (>0.3 mg/kg, s.c.) significantly disrupted PPI, accompanied by the suppression of c-Fos expression in lateral globus pallidus induced by PPI. Furthermore, acute treatment with METH and MK-801 stimulated c-Fos expression in the caudal pontine reticular nucleus (PnC) in mice subjected to the PPT test, although PPI alone had no effect on c-Fos expression. Repeated treatment with 1 mg/kg METH for 7 days, which did not affect PPI acutely, showed similar effects on PPI and c-Fos expression to acute treatment with METH (3 mg/kg). Baclofen dose-dependently ameliorated PPI impairment induced by acute treatment with METH (3 mg/kg) and MK-801 (1 mg/kg), and decreased METH- and MK-801-stimulated c-Fos expression in PnC to the basal level. These results suggest that dysfunction of pallidotegmental neurons is involved in PPI disruption caused by METH and MK-801 in mice. $GABA_B$ receptor may constitute a putative target in treating neuropsychiatric disorders with sensorimotor gating deficits, such as schizophrenia and METH psychosis.

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INTRODUCTION

Prepulse inhibition (PPI) of the startle reflex is commonly viewed as an operational measure of a process called 'sensorimotor gating,' by which excess or trivial stimuli are screened or 'gated out' of awareness (Hoffman and Ison, 1980; Braff and Geyer, 1990). PPI is the reduction of the startle response, which occurs when a weak sensory stimulus (prepulse) is presented several hundred milliseconds before a sudden intense stimulus (pulse) (Ison and Hammond, 1971; Swerdlow *et al*, 2001; Fendt *et al*, 2001; Yeomans *et al*, 2006).

Like the startle reflex itself, PPI is also a cross-species phenomenon. Deficits in PPI are observed in patients suffering from certain neuropsychiatric disorders such as schizophrenia. (Braff *et al*, 1978; Castellanos *et al*, 1996). Deficits of PPI in schizophrenia can be mimicked in rodents by treatment with psychostimulants such as methamphetamine (METH) (Dai *et al*, 2004) and non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonists such as MK-801 and phencyclidine (Curzon and Decker, 1998).

In a previous study, we investigated the neuronal circuits underlying the control of PPI of the acoustic startle reflex in mice by quantifying changes in the expression of c-Fos

*Correspondence: Dr K Yamada, 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 2682, E-mail: kyamada@med.nagoya-u.ac.jp
[7]These authors contributed equally to this work.
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protein in various areas of the brain in mice exposed to either background noise, pulses without prepulses, pulses with prepulses, or prepulses without pulses. c-Fos immunohistochemistry revealed that the lateral globus pallidus (LGP) was activated by prepulses whereas activation of the caudal pontine reticular nucleus (PnC) evoked by startle pulses was inhibited by prepulses. Double-immunostaining revealed that c-Fos-positive cells in LGP following prepulse trials were GABAergic neurons. Furthermore, it was demonstrated that fluoro-gold infusion into PnC and the pedunculopontine tegmental nucleus (PPTg) retrogradely labeled neurons in PPTg and LGP, respectively. Inactivation of LGP by lidocaine resulted in impairment of PPI of the startle reflex. Microinjection with phaclofen, a $GABA_B$ receptor antagonist, into PPTg significantly impaired PPI, whereas picrotoxin, a $GABA_A$ receptor antagonist, had no effect. Based on these findings, we have proposed the hypothesis that neural circuits containing pallidotegmental GABAergic neurons play a crucial role in PPI of the acoustic startle reflex via $GABA_B$ receptors in mice (Takahashi *et al*, 2007).

GABA is the most important inhibitory neurotransmitter in the brain, and deficits of the GABAergic system, including the reduced number of parvalbumin-containing GABAergic interneurons, have been documented in the prefrontal cortex of patients with schizophrenia (Benes and Berretta, 2001; Lewis *et al*, 2001). Previous studies have indicated that the reduction of GABAergic interneurons in schizophrenia is paralleled by increased $GABA_A$ receptor binding in the prefrontal cortex (Benes *et al*, 1996). In contrast, a marked reduction of $GABA_B$ receptors was reported in the hippocampus and prefrontal cortex (Mizukami *et al*, 2000; Ishikawa *et al*, 2005). Although the role of $GABA_B$ receptors in schizophrenia and related disorders is still poorly understood, recent pharmacologic studies have demonstrated that baclofen, a $GABA_B$ receptor agonist, prevents PPI deficits in rodents (Bortolato *et al*, 2004, 2007).

In this study, we investigated whether PPI disruption caused by METH and MK-801 is associated with the dysfunction of pallidotegmental neurons in mice, by quantifying changes in c-Fos expression in LGP and PnC after the PPI test. Furthermore, we examined the effect of baclofen on METH- and MK-801-induced disruption of PPI of the startle reflex in mice.

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 ($23 \pm 1^{\circ}C$, $50 \pm 5\%$ humidity), with a 12 h light-dark cycle (lights on at 0900 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 1000 and 1700 hours.

All animal care and use were in accordance with the National Institutes 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), MK-801 (Sigma-Aldrich Co., St Louis, MO), and baclofen (Sigma-Aldrich) were dissolved in physiological saline and administered at a volume of 0.1 ml/10 g body weight.

For the acute treatment test, mice were given a subcutaneous (s.c) injection of saline, METH (1 or 3 mg/kg), or MK-801 (0.3 or 1 mg/kg), 10 min before the PPI test. Baclofen (1 or 5 mg/kg, s.c.) was pretreated 15 min before METH and MK-801 treatments. For the repeated METH treatment test, animals were injected with METH (1 mg/kg, s.c.) once a day for 7 days. The animals were subjected to the PPI test 10 min, 3, 7 or 14 days after the last administration of METH. Control animals were given saline under the same schedule as for repeated administration of METH.

PPI Test

A standard startle chamber 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 i.d., 50 mm o.d.), placed in a sound-attenuated text 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 non-stimulated 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. Non-stimulated control mice were used without being subjected to PPI trials. The PPI test has three continuous sessions: (1) five startle trials, (2) 10 startle trials, 10 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 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 intensities 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 10 times. During the no-stimulus trial, no stimulus was presented but the movement of the animal was scored. 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 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 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 (Ellenbroek *et al*, 2002; Takahashi *et al*, 2007).

Fos Immunohistochemistry

c-Fos immunostaining was performed as described previously (Takahashi *et al*, 2007). As Fos expression was shown to occur from 1 to 4 h after a single short stimulation (Herdegen and Leah, 1998), animals were deeply anesthetized with sodium pentobarbital (50 mg/kg) 2 h after the PPI test. Alternatively, mice were treated with saline, METH (3 mg/kg, s.c.), or MK-801 (1 mg/kg, s.c.) 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 μm) 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 4°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, Jene, 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). We selected three different sections from each animal, which showed typical and average responses in the PPI test, and defined the region of interest (ROI), 500 × 500 pixels (168 × 168 μm), using the software in both 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 on the border of ROI. c-Fos-positive cells were counted three times with differential ROI in the same section. The average of the resulting nine determinations of the c-Fos-positive cell numbers was used for statistical analysis (Takahashi *et al*, 2007). c-Fos-positive cells were counted by an individual blind to the treatment conditions. In the acute drug treatment test, selected areas were as follows: LGP, PnC, medial prefrontal cortex (mPFC), somatosensory cortex (SC), core and shell of the nucleus accumbens (NAc). In the repeated METH treatment test, only LGP and PnC were selected.

Statistical Analysis

Results are expressed as the mean ± SE. The significance of differences was determined by one-way analysis of variance (ANOVA) in experiments counting c-Fos-positive cells and by repeated measures ANOVA in PPI response measurement, followed by the *post hoc* Bonnferroni/Dunn test. Unpaired *t*-test was used for two-group comparisons. *p*-values less than 0.05 were regarded as statistically significant.

RESULTS

Effect of Acute Treatment with METH and MK-801 on PPI of the Acoustic Startle Reflex in Mice

Figure 1 shows the effect of acute METH (1 and 3 mg/kg, s.c.) and MK-801 (0.3 and 1 mg/kg, s.c.) treatment on PPI in mice. For the effect of METH, repeated measures of ANOVA revealed significant effects of METH treatment ($F(2,31) = 8.711$, $p < 0.01$) and prepulse intensity ($F(3,93) = 11.023$, $p < 0.001$), but not their interaction ($F(6,93) = 0.908$, $p > 0.05$). METH at a dose of 3 mg/kg significantly reduced PPI compared with the saline-treated control group ($p < 0.001$) (Figure 1a). For the effect of MK-801, there were significant effects of MK-801 treatment ($F(2,29) = 8.337$, $p < 0.01$) and prepulse intensity ($F(3,87) = 14.646$, $p < 0.001$), but not their interaction ($F(6,87) = 0.224$, $p > 0.05$). MK-801 dose-dependently reduced PPI compared with the saline-treated control group ($p < 0.01$) (Figure 1c). METH had no effect on the startle amplitude (Figure 1b), whereas MK-801 at 0.3 mg/kg significantly increased the startle amplitude ($F(2,29) = 7.418$, $p < 0.01$) (Figure 1d).

PPI-Associated Changes in c-Fos Expression in the LGP and PnC of Mice Treated with Acute METH and MK-801

To detect 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, METH (3 mg/kg, s.c.), or MK-801 (1 mg/kg, s.c.). Representative photomicrographs of c-Fos staining in the LGP and PnC after the PPI test are shown in Figures 2 and 3, respectively. Quantitative analysis of c-Fos staining indicated a significant difference in the number of c-Fos-positive cells in the LGP ($F(3,14) = 60.936$, $p < 0.001$) (Figure 4a). There was a significant increase in the number of c-Fos-positive cells in saline-treated control mice subjected to the PPI test ($n = 4$) compared with non-stimulated mice ($n = 5$) ($p < 0.001$). The number of c-Fos-positive cells in the LGP of mice treated with METH ($n = 4$) or MK-801 ($n = 5$) was significantly less than that in saline-treated control mice subjected to the PPI test ($p < 0.001$).

A significant change in c-Fos expression was also observed in the PnC ($F(3,14) = 14.43$, $p < 0.001$) (Figure 4b). There was no difference in the number of c-Fos-positive cells between non-stimulated mice ($n = 4$) and saline-treated control mice subjected to the PPI test ($n = 4$). The number of c-Fos-positive cells in the PnC of mice treated with METH ($n = 4$) or MK-801 ($n = 4$) was significantly increased compared with that in saline-treated control mice ($p < 0.001$).

Furthermore, we analyzed the changes of c-Fos expression in the mPFC, SC, NAc shell, and NAc core of mice treated with saline ($n = 4$), METH (3 mg/kg, s.c., $n = 4$), or MK-801 (1 mg/kg, s.c., $n = 4$) after the PPI test (Figure 5). Significant differences in c-Fos expression were observed in

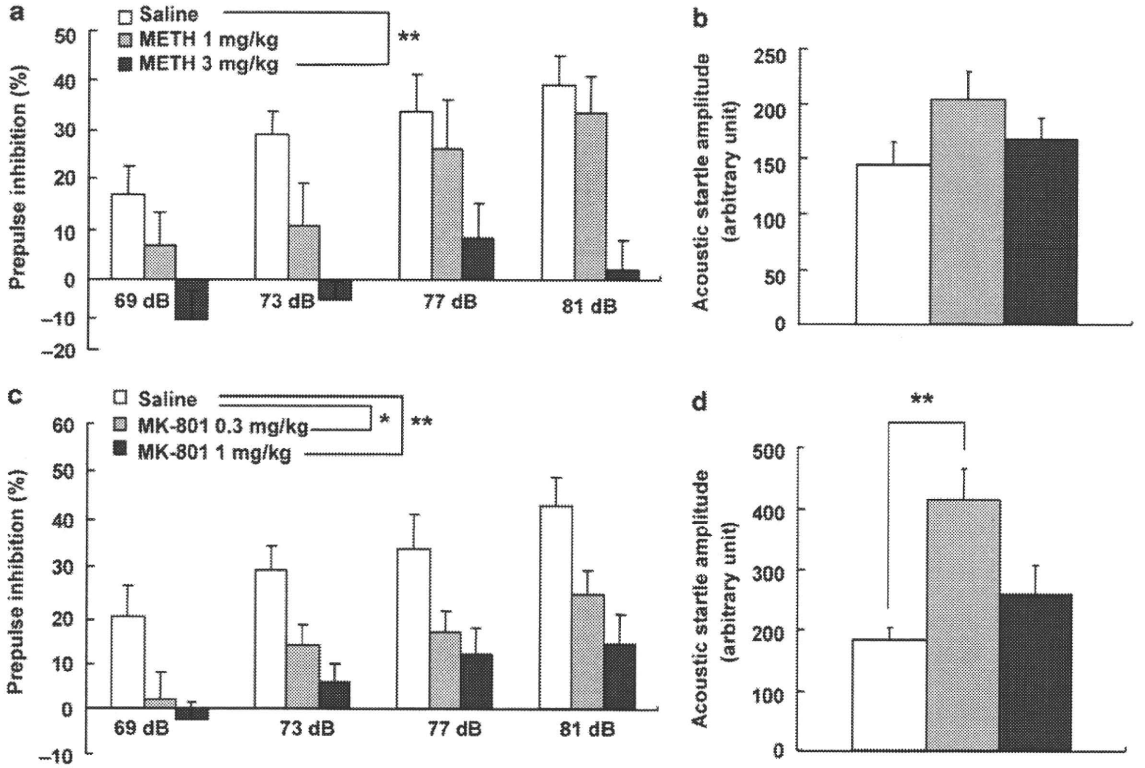

Figure 1 Effects of acute METH and MK-801 treatment on PPI in mice. Mice were treated with saline, METH (1 and 3 mg/kg, s.c.) (a, b), or MK-801 (0.3 and 1 mg/kg, s.c) (c, d) 10 min before the PPI test. (a, c) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (b, d) Acoustic startle amplitude as measured in trials without a prepulse. Values indicate the mean ± SE (a, b: saline, $n=11$, METH 1 mg/kg, $n=11$, METH 3 mg/kg, $n=12$; c, d: saline, $n=9$, MK-801 0.3 mg/kg, $n=12$, MK-801 1 mg/kg, $n=11$). $^{*}p<0.01$, $^{**}p<0.001$ *vs* the saline-treated control group (Bonnferroni/Dunn test).

the mPFC ($F(3,12)=7.616$, $p<0.01$), NAc shell ($F(3,12)=11.047$, $p<0.001$), and NAc core ($F(3,12)=21.033$, $p<0.001$), whereas there were no significant differences in the SC. There was no difference in the number of c-Fos-positive cells in the mPFC, SC, NAc shell, and NAc core between non-stimulated mice and saline-treated control mice subjected to the PPI test. The numbers of c-Fos-positive cells in the mPFC ($p<0.01$) and NAc core ($p<0.01$) of the METH-treated group were significantly increased compared with those in the saline-treated control group. In contrast, a significant decrease in the number of c-Fos-positive cells was observed in the NAc shell of the MK-801-treated group compared with the saline-treated control group ($p<0.01$). Thus, the effects of METH and MK-801 on the behavioral responses of mice in the PPI test paralleled with the neuronal activity in the LGP, raising the possibility that LGP may be critical for the PPI disruption induced by METH and MK-801.

As a control experiment, we measured the effects of METH (3 mg/kg, s.c.) and MK-801 (1 mg/kg, s.c.) on c-Fos expression in various brain areas of mice that were not subjected to the PPI test (Supplementary Figure S1). Acute METH treatment had no effect on c-Fos expression in the LGP and PnC, but significantly increased the expression in the mPFC, SC, NAc shell, and NAc core ($p<0.01$). Similarly, acute MK-801 treatment did not affect c-Fos expression in the LGP and PnC, while it increased the number of Fos-positive cells in the mPFC and SC ($p<0.001$). From this control experiment, it is obvious that the effects of METH and MK-801 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.

Effect of Repeated Treatment with METH on PPI of the Acoustic Startle Reflex in Mice

Figure 6 shows the effect of single and repeated treatment with METH (1 mg/kg, s.c.) on PPI in mice. Repeated measures of ANOVA revealed significant effects of repeated METH treatment ($F(2,18)=9.152$, $p<0.01$) and prepulse intensity ($F(3,54)=18.311$, $p<0.001$), but not their interaction ($F(6,54)=1.804$, $p>0.05$). Repeated, but not single, METH treatment induced a significant impairment of PPI of the startle reflex compared with saline treatment ($p<0.01$) (Figure 6a). Acute and repeated METH treatment had no effect on the startle amplitude (Figure 6b).

Repeated METH-induced PPI disruption was observed after the withdrawal of METH for at least 7 days without any changes in the startle response. There were significant effects of repeated METH treatment ($F(1,18)=9.101$, $p<0.01$), prepulse intensity ($F(3,54)=49.709$, $p<0.001$), and their interaction ($F(3,54)=3.7$, $p<0.05$) ($n=10$ in each group). However, the disruptive effect of repeated METH treatment on PPI disappeared after withdrawal for 14 days (data not shown).

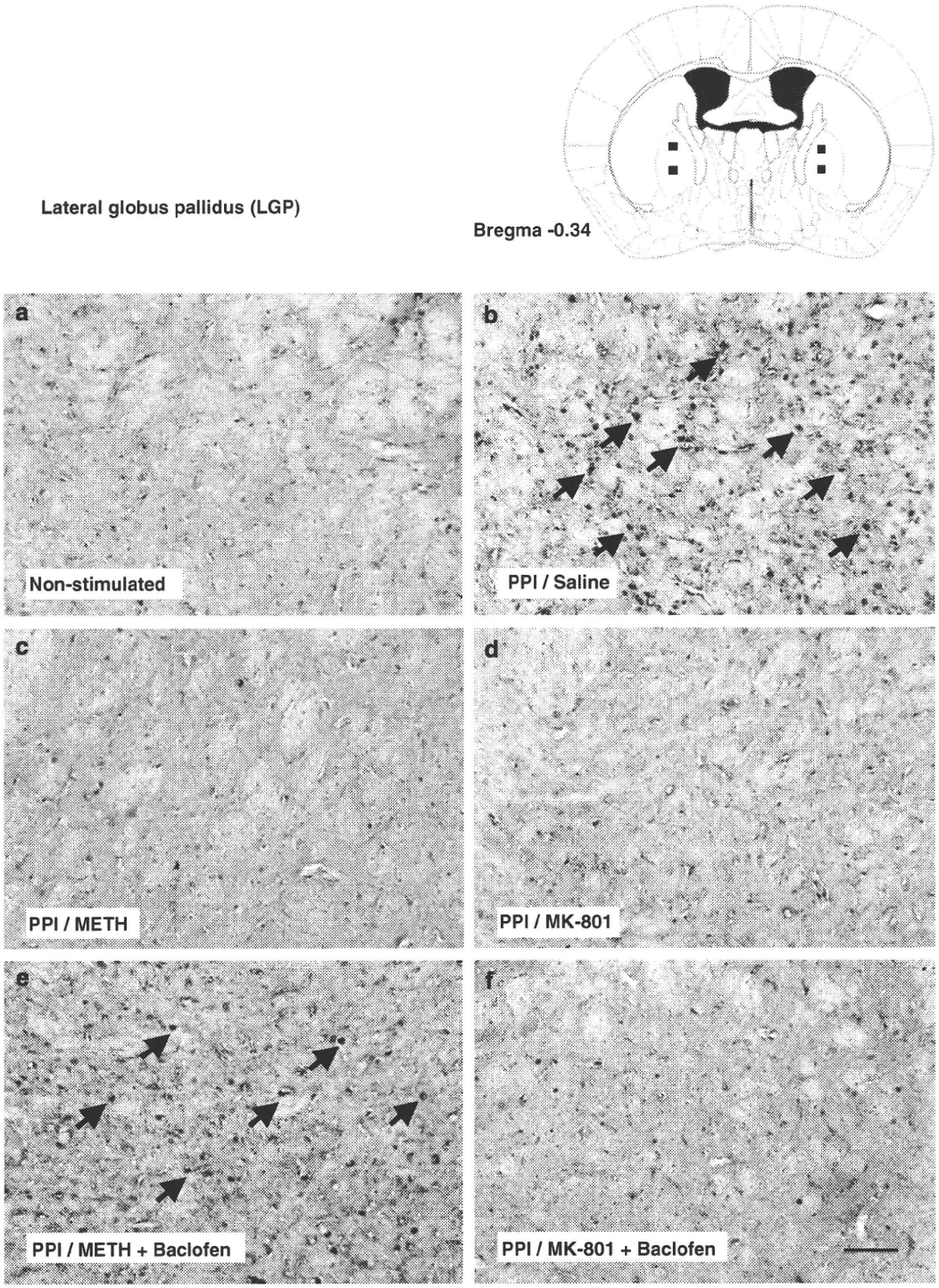

Figure 2 Representative photomicrographs of c-Fos immunostaining in LGP after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group, a). Alternatively, mice were subjected to the PPI test after pretreatment with saline (b), METH (3 mg/kg, c), MK-801 (1 mg/kg, d), and baclofen (5 mg/kg) 15 min before METH (e) or MK-801 (f) treatment. Scale bar, 100 μm. Arrows indicate typical c-Fos-positive cells.

PPI-Associated Changes in c-Fos Expression in the LGP and PnC of Mice with Repeated METH Treatment

To examine the effects of repeated METH 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, single, or repeated METH after the PPI test (Figure 7). A significant difference in the number of c-Fos-positive cells was observed in LGP ($F(3,13) = 117.265$, $p < 0.001$). There was a significant increase in c-Fos expression in saline-treated control mice subjected to the PPI test ($n = 4$) compared with non-stimulated mice ($n = 5$) ($p < 0.001$). In contrast, the number of c-Fos-positive cells in the LGP of mice with repeated

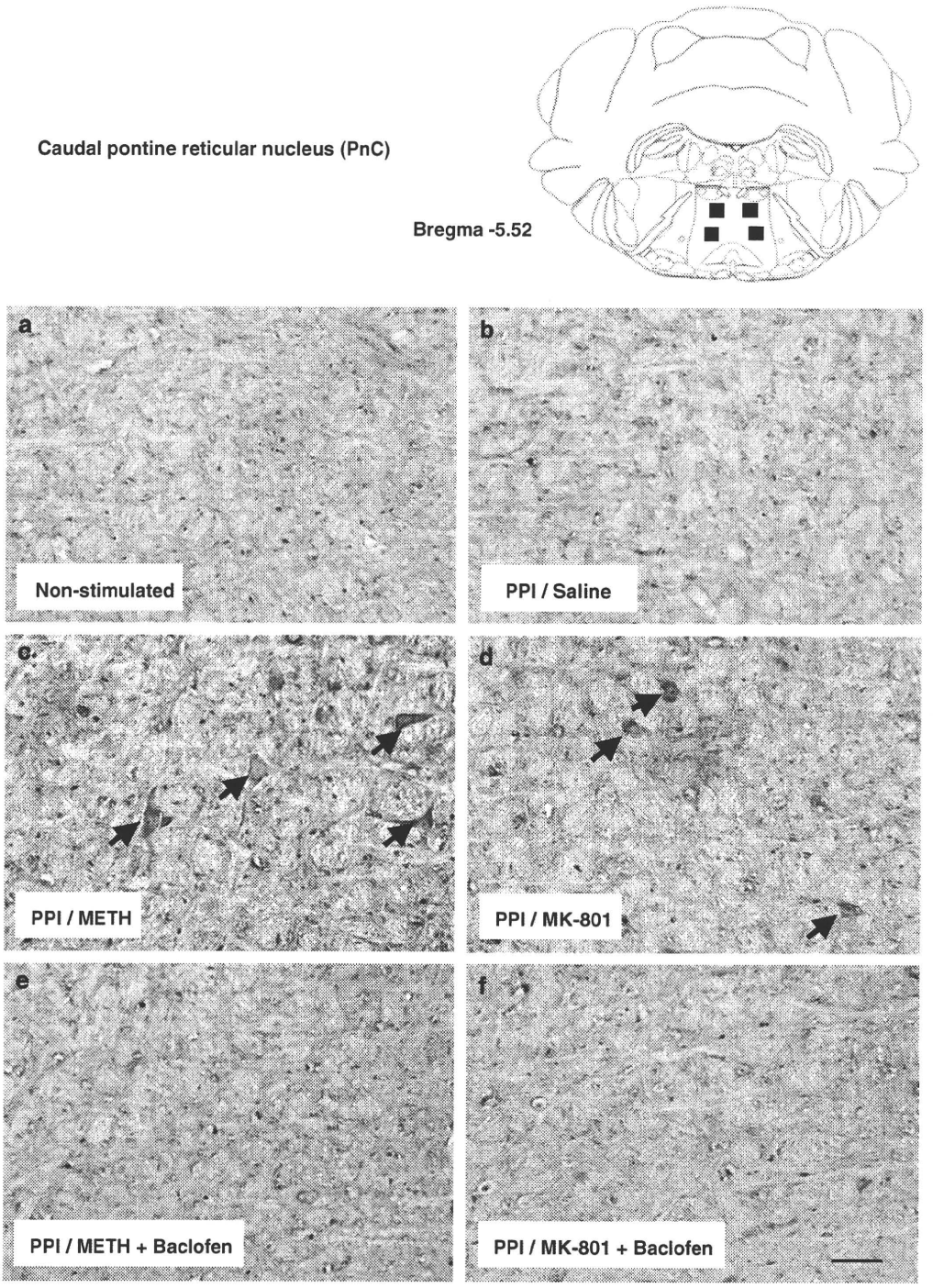

Figure 3 Representative photomicrographs of c-Fos immunostaining in PnC after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group, a). Alternatively, mice were subjected to the PPI test after pretreatment with saline (b), METH (3 mg/kg, c), MK-801 (1 mg/kg, d), and baclofen (5 mg/kg) 15 min before METH (e) or MK-801 (f) treatment. Scale bar, 100 μm. Arrows indicate typical c-Fos-positive cells.

METH treatment ($n=4$) was significantly lower than that in saline-treated control mice ($p<0.001$). There was a slight, but significant decrease in c-Fos expression in the acutely METH-treated group ($n=4$) compared with the saline-treated control group.

A significant difference in c-Fos expression was also observed in PnC ($F(3,16)=13.217$, $p<0.001$). There was no difference in c-Fos expression between non-stimulated mice ($n=5$) and saline-treated control mice ($n=5$) subjected to the PPI test; however, c-Fos expression in repeated ($n=5$), but not acute ($n=5$), METH-treated mice was significantly increased compared with saline-treated control mice ($p<0.001$).

Mice exhibited normal PPI after withdrawal from METH for 14 days and no changes in c-Fos expression were observed in both LGP and PnC as compared with saline-treated control mice (unpaired *t*-test, data not shown).

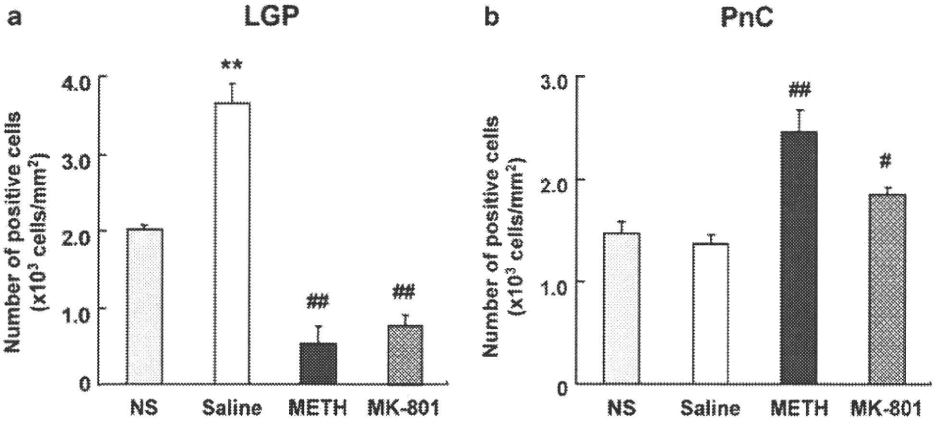

Figure 4 Effects of acute METH and MK-801 treatment on changes in c-Fos expression in LGP (a) and PnC (b) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group: NS). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg), and MK-801 (1 mg/kg). Values indicate the mean ± SE. $^{**}p<0.001$ *vs* the non-stimulated group. $^{\#}p<0.01$, $^{\#\#}p<0.001$ *vs* the saline-treated control group (*post hoc* test).

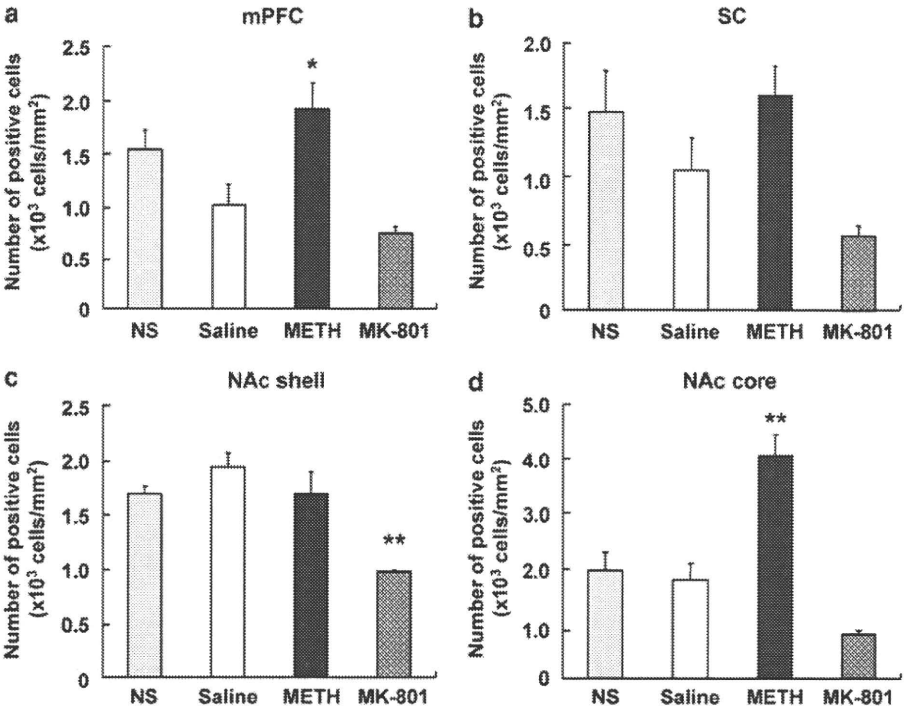

Figure 5 Effects of acute METH and MK-801 treatment on c-Fos expression in the mPFC (a), SC (b), NAc shell (c), and NAc core (d) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group: NS). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg), and MK-801 (1 mg/kg). Values indicate the mean ± SE (all groups, $n=4$). $^{*}p<0.01$, $^{**}p<0.001$ *vs* the saline-treated control group (*post hoc* test).

Effects of Baclofen on METH- and MK-801-Induced PPI Impairment in Mice

Baclofen (1 and 5 mg/kg, s.c.), a $GABA_B$ receptor agonist, dose-dependently reversed the impairment of PPI induced by METH (3 mg/kg, s.c.) and MK-801 (1 mg/kg, s.c.) without any effects on the startle amplitude (Figure 8). For the effect of baclofen on METH-induced PPI disruption, there were significant effects of drug treatment ($F(3,26)=6.73$, $p<0.01$) and prepulse intensity ($F(3,78)=33.278$, $p<0.001$), but not their interaction ($F(9,78)=0.550$, $p>0.05$). METH significantly reduced PPI compared with the saline-treated control group ($p<0.01$). Baclofen, at a dose of 5 mg/kg, significantly ameliorated PPI impairment in the METH-treated group ($p<0.001$) (Figure 8a). For the effect of baclofen on MK-801-induced PPI disruption, there were significant effects of drug treatment ($F(3,24)=4.852$, $p<0.01$) and prepulse intensity ($F(3,72)=49.336$, $p<0.001$), but not their interaction ($F(9,72)=0.1467$, $p>0.05$). MK-801 significantly reduced PPI compared with the saline-treated control group ($p<0.01$). Baclofen at a dose of 5 mg/kg significantly

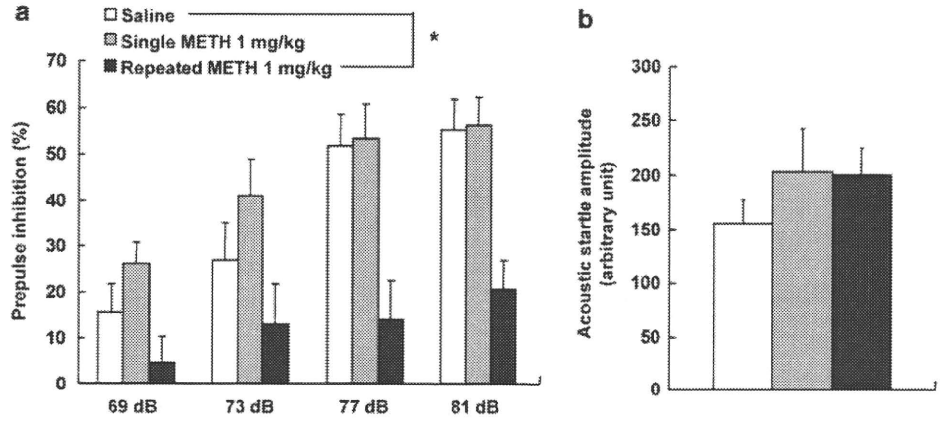

Figure 6 Effects of single and repeated METH treatment on PPI in mice. Mice were treated with METH (1 mg/kg, s.c.) acutely 10 min before the PPI test. Alternatively, METH (1 mg/kg) was repeatedly injected into mice for 7 days, and the animals were subjected to the PPI test 10 min after the last injection. (a) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (b) Acoustic startle amplitude as measured in trials without a prepulse. Values indicate the mean ± SE (a, b: all groups, $n=7$). *$p<0.01$ *vs* the saline-treated control group (Bonnferroni/Dunn test).

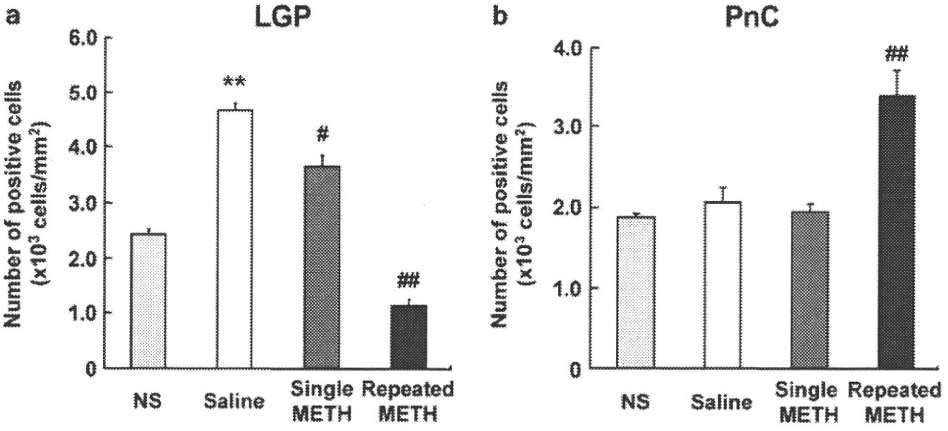

Figure 7 Effects of single and repeated METH treatment on changes in c-Fos expression in the LGP (a) and PnC (b) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group: NS). Alternatively, mice were subjected to the PPI test 10 min after pretreatment with acute saline, METH (1 mg/kg, s.c.), and repeated METH treatment for 7 days. Values indicate the mean ± SE (a: non-stimulated, $n=5$, other groups, $n=4$; b: all groups, $n=5$). **$p<0.001$ *vs* the non-stimulated group. $^{\#}p<0.01$, $^{\#\#}p<0.001$ *vs* the saline-treated control group (Bonnferroni/Dunn test).

increased PPI compared with the MK-801-treated group ($p<0.01$) (Figure 8c). Acute treatment with baclofen alone (1 and 5 mg/kg) had no effect on PPI or the startle response in mice (data not shown).

Effects of Baclofen on the METH- and MK-801-Induced Changes in c-Fos Expression in the LGP and PnC of Mice Subjected to the PPI Test

To study the effects of baclofen (5 mg/kg, s.c.) on METH- and MK-801-induced changes in pallidotegmental neural activation in mice that were subjected to the PPI test, we examined c-Fos expression immunohistochemically in the LGP and PnC of mice after the PPI test. Representative photomicrographs of c-Fos staining in the LGP and PnC are shown in Figures 2 and 3, respectively.

As shown in Figure 9, quantitative analysis of c-Fos staining indicated a significant difference in the number of c-Fos-positive cells in LGP ($F(4,12)=25.589$, $p<0.001$). There was a significant decrease in c-Fos expression in the LGP of mice treated with METH (3 mg/kg, $n=3$) or MK-801 (1 mg/kg, $n=3$) compared with the saline-treated control group ($n=3$) ($p<0.001$). Pretreatment with baclofen significantly increased the number of c-Fos-positive cells in the LGP compared with the METH-treated group ($n=4$) ($p<0.001$). In contrast, baclofen had no effect on MK-801-induced reduction of c-Fos expression in the LGP ($n=4$), although it ameliorated the MK-801-induced disruption of PPI.

A significant effect of baclofen on c-Fos expression was also observed in the PnC ($F(4,12)=25.008$, $p<0.001$). c-Fos expression in the PnC of mice treated with METH ($n=3$) or MK-801 ($n=3$) was significantly increased compared with saline-treated control mice ($n=3$) ($p<0.001$). Both METH ($n=4$)- and MK-801 ($n=5$)-induced increase in the number of c-Fos-positive cells in PnC was suppressed by pretreatment with baclofen ($p<0.001$).

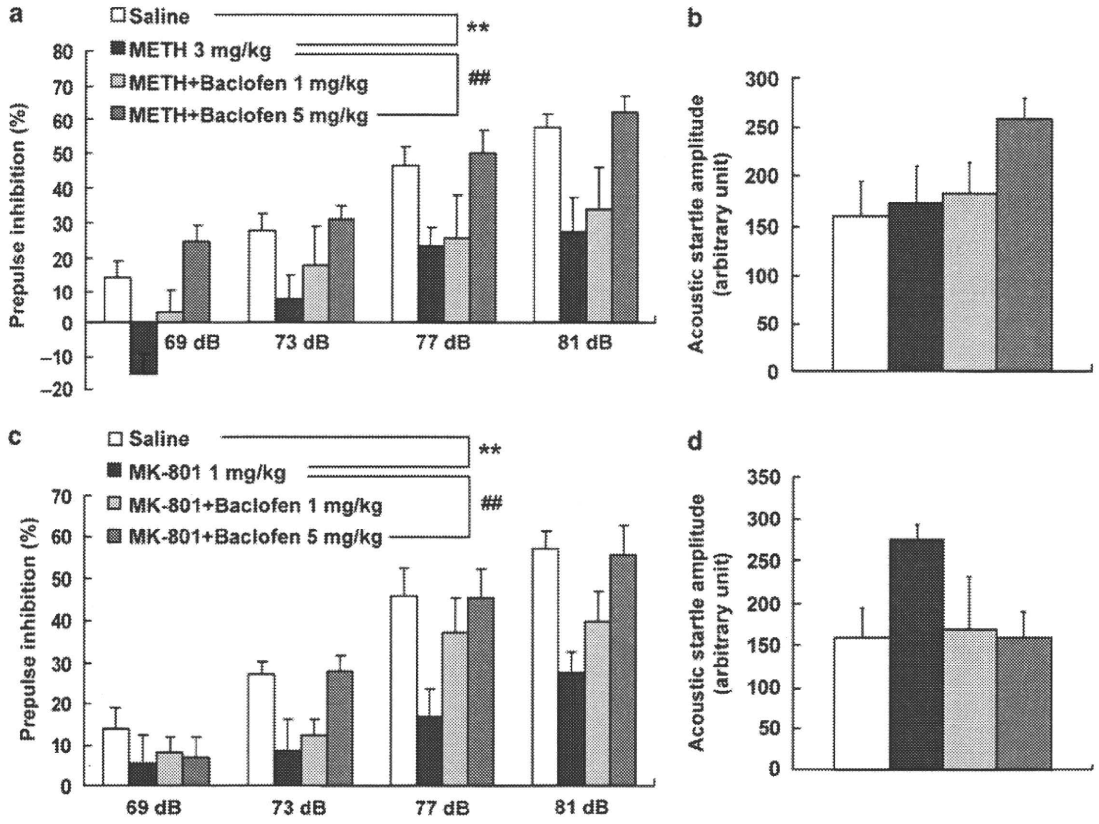

Figure 8 Effects of baclofen on acute METH- and MK-801-induced impairment of PPI in mice. Mice were treated with baclofen (1 and 5 mg/kg, s.c.) 15 min before METH (3 mg/kg, s.c.) (a, b) or MK-801 (1 mg/kg, s.c.) (c, d) treatment. (a, c) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (b, d) Acoustic startle amplitude as measured in trials without a prepulse. Values indicate the mean ± SE (a, b: saline, $n=7$, METH: $n=7$, METH with baclofen 1 mg/kg: $n=8$, METH with baclofen 5 mg/kg: $n=9$; c, d: saline: $n=7$, MK-801: $n=6$, MK-801 with baclofen 1 mg/kg: $n=8$, MK-801 with baclofen 5 mg/kg: $n=7$). $^{**}p<0.001$ *vs* the saline-treated control group. $^{\#\#}p<0.01$ *vs* the METH- or MK-801-treated group (Bonnferroni/Dunn test).

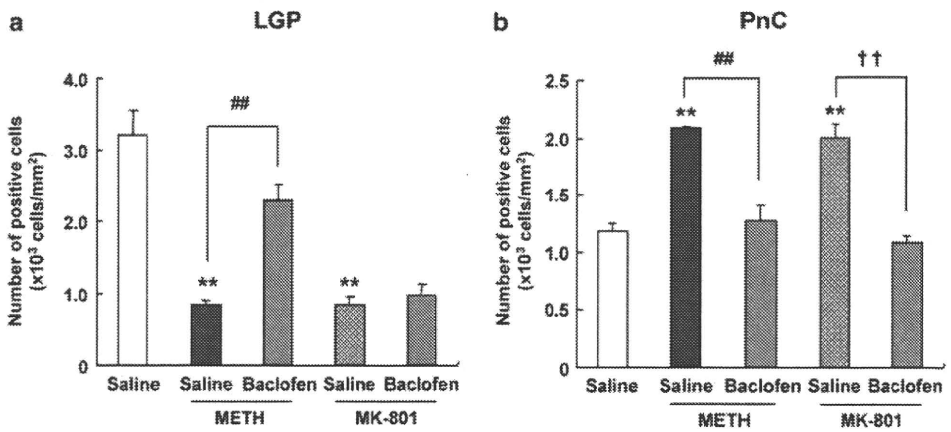

Figure 9 Effects of baclofen on METH- and MK-801-induced changes in c-Fos expression in LGP (a) and PnC (b) after the PPI test. Mice were restricted to the PPI test cage, but not subjected to the PPI test (non-stimulated group). Alternatively, mice were subjected to the PPI test after pretreatment with saline, METH (3 mg/kg, s.c.), and MK-801 (1 mg/kg, s.c.), and baclofen (5 mg/kg, s.c.) followed by METH or MK-801. Values indicate the mean ± SE (a: saline, METH and MK-801, $n=3$, other groups, $n=4$; b: saline, METH and MK-801, $n=3$, METH with baclofen, $n=4$, MK-801 with baclofen, $n=5$). $^{**}p<0.001$ *vs* the saline-treated control group. $^{\#\#}p<0.001$ *vs*. the METH-treated group. $^{\dagger\dagger}p<0.001$ *vs* the MK-801-treated group (Bonnferroni/Dunn test).

DISCUSSION

The fast excitatory pathway of the acoustic startle system involves serial connections linking the auditory nerve, cochlear root neurons, PnC, and spinal motor neurons (Lee *et al*, 1996). We have previously demonstrated that pulse stimulus increased c-Fos expression in the PnC, which was attenuated by a weak prepulse before pulse stimulus in the PPI test (Takahashi *et al*, 2007). Our findings are consistent with data indicating that PnC neurons are

markedly inhibited by an acoustic prepulse in mice (Willot *et al*, 1994; Carlson and Willot, 1998). In this study, an increase in the number of c-Fos-positive cells in the PnC after the PPI test was evident only when PPI was disrupted by acute METH or MK-801 treatment as well as repeated METH treatment. These results suggest that the fast excitatory pathway of the acoustic startle system, including PnC, is activated even in the presence of prepulse stimulus if dopaminergic synaptic transmission is potentiated by METH or glutamatergic neurotransmission through NMDA receptors is blocked by MK-801, which results in the disruption of PPI of the acoustic startle reflex in mice.

We have also demonstrated that GABAergic neurons in LGP, which project directly to PPTg, are activated by prepulse stimulus regardless of startle pulse stimuli (Takahashi *et al*, 2007). Since the localized inactivation of LGP by lidocaine markedly reduced PPI without affecting the startle response induced by a pulse stimulus, it is suggested that GABAergic neurons in LGP play a crucial role in the regulation of PPI of the startle reflex. Moreover, we suggest that pallidotegmental GABAergic neurons act as an interface between the brainstem PPI-mediating areas and forebrain PPI-regulating areas (Takahashi *et al*, 2007). In this study, we demonstrated that PPI disruption induced by treatment with METH or MK-801 was accompanied by the dysfunction of LGP, which was manifested by a reduction of c-Fos expression in LGP after the PPI test. Although the causal relation between the phenomena is unclear, taking our previous findings into consideration, it is reasonable to assume that both METH and MK-801 disrupt PPI of the startle reflex in mice by inhibiting the activation of pallidotegmental GABAergic neurons evoked by a prepulse stimulus. Further studies are required to test this assumption. In particular, it remains to be determined how METH and MK-801 inhibited neural activation in LGP evoked by prepulse stimulus.

We have previously reported that repeated, but not acute, treatment with METH at a low dose (1 mg/kg) in mice induces long-lasting impairment of recognition memory, which is associated with dysfunction in the prefrontal cortex (Kamei *et al*, 2006). Because of the therapeutic effect of clozapine, but not haloperidol, on repeated METH-induced recognition memory impairment, we have proposed that this animal model may be useful for screening potential antipsychotics and studying the pathophysiology of cognitive deficits in schizophrenic patients as well as METH abusers. In patients suffering from schizophrenia and other psychiatric disorders, PPI is disrupted (Braff *et al*, 1978; Swerdlow *et al*, 1994, 1995; Castellanos *et al*, 1996). Accordingly, to further study the face validity of the animal model with repeated METH treatment, we measured the PPI of the acoustic startle reflex in mice that had previously been treated with METH (1 mg/kg) for 7 days. Mice with repeated METH treatment exhibited lasting impairment of PPI without any changes in startle amplitude, and PPI disruption was associated with the dysfunction of LGP and hyperactivation of PnC. These results are consistent with the clinical finding of whole-brain functional magnetic resonance imaging in groups of healthy subjects and schizophrenic patients: significant activation in the globus pallidus/putamen extending to the hippocampus and thalamus during the prepulse experiment was demonstrated in healthy subjects, but the patients showed a lack of activation in subcortical and limbic areas (Kumari *et al*, 2003).

We demonstrated in this study that baclofen, a $GABA_B$ receptor agonist, ameliorated both METH- and MK-801-induced PPI impairment at doses that had no effect on PPI and the startle response by itself. Consistent with our findings, it was reported that baclofen reversed the reduction in PPI induced by MK-801, but not by apomorphine (a direct dopamine receptor agonist), in rats (Bortolato *et al*, 2004), and that baclofen and clozapine, but not haloperidol, improved spontaneous PPI deficit in mice (Bortolato *et al*, 2007). In addition, a recent study (Wolf *et al*, 2007) demonstrated that acute and subchronic treatment with clozapine failed to ameliorate PPI deficits in mice. Taken together, $GABA_B$ receptor agonists may be more useful for the treatment of schizophrenic patients and METH abusers with sensorimotor gating deficit than clozapine and other antipsychotic drugs.

The ameliorating effect of baclofen on PPI was associated with the normalization of PnC hyperactivation. These results are consistent with our previous finding that microinjection of a $GABA_B$ receptor antagonist, phaclofen, but not a $GABA_A$ receptor antagonist, picrotoxin, into the PPTg impaired PPI in mice (Takahashi *et al*, 2007). Similarly, Koch *et al* (2000) indicated that microinjection of phaclofen, but not picrotoxin, into the PnC reduced PPI in rats, and that nigroreticular GABAergic projection-interacting $GABA_B$ receptors are involved in sensorimotor gating.

Baclofen increased the number of c-Fos-positive cells in the LGP of METH-treated mice subjected to the PPI test, whereas it had no effect on MK-801-treated mice. Thus, it is possible that distinct but overlapping neural circuits may be involved in the ameliorating effect of baclofen on METH- and MK-801-induced PPI deficits. For example, baclofen may ameliorate METH-induced PPI impairment, at least in part, through the activation of pallidotegmental GABAergic neurons, whereas it may improve MK-801-induced PPI impairment at PnC levels. Previous studies demonstrated that baclofen reduced the reinforcing effects of the main substances of abuse such as cocaine nicotine, heroine, 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), and to block the development and expression of sensitization to the locomotor stimulation effect of amphetamine (Bartoletti *et al*, 2004, 2005). It is possible, therefore, that baclofen may ameliorate METH-induced PPI impairment by, at least in part, stabilizing the firing of dopamine neurons, which have been demonstrated to be of critical importance in the control of sensorimotor gating (Mansbach *et al*, 1988; Swerdlow and Geyer, 1998). It is clear that further studies are required to clarify the neurobiological mechanisms underlying the PPI deficit induced by METH and MK-801 as well as the restorative effect of baclofen on PPI disruption.

In conclusion, we demonstrated that PPI disruption caused by METH and MK-801 is associated with the dysfunction of pallidotegmental neurons in mice. Repeated METH-treated mice, an animal model for cognitive deficits

in METH abuse and schizophrenia, exhibit PPI impairment. Furthermore, it was demonstrated that baclofen, a $GABA_B$ receptor agonist, is effective in ameliorating PPI disruptions caused by METH and MK-801, suggesting that $GABA_B$ receptors may constitute a putative new target in treating neuropsychiatric disorders with sensorimotor gating deficits, such as schizophrenia and METH psychosis.

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DISCLOSURE/CONFLICT OF INTEREST

There are no conflicts of interest.

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特集：統合失調症の病態進行・難治化と動物モデル

31-40

難治性統合失調症の動物モデルと治療薬開発

古関竹直[1]，毛利彰宏[1]，村井里菜[1]
永井拓[2]，野田幸裕[3]，鍋島俊隆[1]

Key words：schizophrenia, phencyclidine (PCP), glutamatergic neuron, dopaminergic neuron, aripiprazole, galantamine, Disrupted-in-schizophrenia-1 (DISC1)

1．はじめに

統合失調症は，主に思春期以降に発症する慢性・進行性の精神疾患であり，幻覚や妄想，まとまりに欠ける会話や行動，感情の平板化，意欲の低下，認知障害などを主訴とする[1]。意欲の低下などの陰性症状や認知障害は，第一世代抗精神病薬に抵抗性であり，難治性の統合失調症として捉えられている。新たな治療戦略を考えるためには，臨床研究に加え，適切な病態モデル動物を用いた神経機能の解明および新規治療薬の評価などの基礎研究が必要である。非競合的N-methyl-D-aspartate（NMDA）受容体拮抗薬のフェンシクリジン（PCP）は，薬物依存者に統合失調症に酷似した精神症状（PCP精神病）を惹起することから，統合失調症はNMDA受容体機能の低下が関与しているという「グルタミン酸作動性神経系機能低下仮説」が提唱されている[2]。PCP連続投与動物モデルは，陽性症状の指標となる自発運動量の増加や常同行動，陰性症状の指標となる社会性行動の低下や強制水泳試験における意欲の低下および認知障害の指標となる放射状迷路試験における作業記憶障害や水探索試験における注意障害など，臨床症状を反映した多様な行動異常を示すことから，包括的な統合失調症のモデルとして提唱されている[3)4)5)6)]。当教室においても，PCP連続投与動物モデルを用いた統合失調症の病態解明および新規抗精神病薬の評価を行っている。一方，統合失調症の病因には，遺伝要因が関与していることが家系研究，双生児研究，養子研究などから示唆されており，多くの候補遺伝子が見出されている。それらの候補遺伝子を改変させたマウスの中には，統合失調症様の精神行動障害を示し，モデル動物としての有用性が報告されているものがある[7)8)9)]。細胞移動および神経突起の伸長など，

Schizophrenic animal models and evaluation of antipsychotics

1）名城大学大学院薬学研究科薬品作用学教室〔〒468-8503 名古屋市天白区八事山150〕Takenao Koseki, Akihiro Mouri, Rina Murai, Toshitaka Nabeshima：Department of Chemical Pharmacology, Meijo University Graduate School of Pharmaceutical Sciences, 150, Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan
2）名古屋大学大学院医学系研究科医療薬学・医学部附属病院薬剤部 Taku Nagai：Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine
3）名城大学大学院薬学研究科病態解析学I Yukihiro Noda: Division of Clinical Science in Clinical Practice, Management and Research, Meijo University Graduate School of Pharmaceutical Sciences
【鍋島俊隆　E-mail：tnabeshi@ccmfs.meijo-u.ac.jp】

神経発達期において重要な役割を担っているDisrupted-in-schizophrenia-1（DISC1）は，スコットランドの大家系において転座が認められ，統合失調症関連遺伝子であることが報告されている[10]。当教室において，子宮内エレクトロポレーション法により大脳皮質におけるDISC1の発現を神経発達過程で抑制したKnock DownマウスおよびDISC1 KDマウスを紹介し，これらモデル動物を用いた抗精神病薬の評価について概説する。

2．PCP連続投与マウスに認められる統合失調様症状

1．陽性症状様行動障害

臨床知見において，PCPは幻覚，妄想などの陽性症状に酷似した症状を惹起することが知られている[11]。マウスに低用量のPCP（3mg/kg）を単回投与すると，側坐核の細胞外ドパミン遊離量の増加とともに，自発運動量が増加する[12]。そのため，PCPによる自発運動量の増加は，陽性症状の指標としてよく用いられている。PCP（10mg/kg）を14日間連続投与したマウス（PCP連続投与マウス）に，3日間の休薬後に低用量のPCPを再投与すると，単回投与した時に比べて側坐核のドパミン遊離量と自発運動量が著しく増加する（行動感作）[13]。PCP連続投与における行動感作は，主としてドパミンD2受容体を遮断する第一世代抗精神病薬であるハロペリドール，第二世代抗精神病薬のセロトニン・ドパミン受容体アンタゴニスト（SDA）であるリスペリドン，多受容体作用抗精神病薬（MARTA）であるクロザピンによって抑制される。これらの知見は，臨床において陽性症状に対して第一世代および第二世代抗精神病薬がともに有効であることと一致している。そのため，PCP連続投与動物にみられる行動感作は，統合失調症陽性症状モデルとして有用であると思われる。

2．陰性症状様行動障害とグルタミン酸作動性神経系の機能低下

統合失調症患者に認められる陰性症状として，意欲の低下および社会性行動の低下などが知られている。我々は，統合失調症の陰性症状の評価方法として，強制水泳試験と社会性行動試験を用いている。強制水泳試験では，水を入れた狭いシリンダーにマウスやラットを入れると，最初はそこから逃げようと激しく動くが，やがて水に浮かんでいるだけで動かない状態（無動化あるいは絶望状態）になる。強制水泳試験において，PCP連続投与マウスは無動状態の著しい増強（意欲低下の増強）が認められ，この無動状態の増強は，PCP最終投与3週間後においても認められる[4) 13)]。PCP連続投与による無動状態の増強は，第二世代抗精神病薬であるリスペリドン，クロザピンによって緩解されるが，第一世代抗精神病薬であるハロペリドールでは緩解されない[14]。一方，社会性行動は実験動物においても認められ，別々のケージで飼育され，それまで接触する機会のなかった2匹のマウスやラットを同時に観察ケージに入れると，お互いに匂いを嗅ぐ，毛繕いをする，後をついて歩くといった行動を示す。Sams-Doddはラットを用いた実験で，PCP単回投与において社会性行動の低下が認められるが，覚せい剤投与ではそのような障害が認められないことを報告している[15]。一方，PCP連続投与マウスにおいても社会性行動の低下が認められ，我々の知見では社会性行動の低下はPCP最終投与4週間後においても認められる[5]。PCP連続投与による社会性行動の低下は，第二世代抗精神病薬であるクロザピンによって緩解されるが，第一世代抗精神病薬であるハロペリドールでは緩解されない[5]。臨床知見において，陰性症状に対して第一世代抗精神病薬は無効であるが，第二世代抗精神病薬は有効であることから，PCP連続投与動物は，臨床で認められる陰性症状を反映した難治性統合失調症動物モデルと言える。

統合失調症の病態仮説の一つであるグルタミン酸作動性神経系の機能低下は，PCP連続投与マウスにおいても認められる[16]。マイクロダイアリシス法を用いて細胞外グルタミン酸濃度を測定する

と，PCP連続投与マウスの前頭前皮質において，細胞外グルタミン酸濃度の低下が認められる。また，グルタミン酸の再取り込み能を有し，その細胞外濃度を調節しているグリア型グルタミン酸トランスポーターであるGlial glutamate-aspartate transporter（GLAST）の発現量を免疫組織化学法およびウェスタンブロッティング法により測定すると，PCP連続投与マウスの前頭前皮質において，GLAST発現量の著しい増加が認められる。一方，非特異的グルタミン酸トランスポーター阻害剤であるDL-threo-b-benzyloxyaspartateを前頭前皮質へ微量注入すると，PCP連続投与マウスに認められる強制水泳試験時の無動状態の増強が緩解される。これらの知見は，PCP連続投与により前頭前皮質で増加したGLASTが，細胞外へ遊離したグルタミン酸の量を減少させ，強制水泳試験で認められる意欲低下を惹起していることを示唆している。

グルタミン酸受容体の一つであるNMDA受容体が活性化されると，Ca^{2+}/calmodulin kinase Ⅱ（CaMK Ⅱ）が活性化される。CaMK Ⅱは神経細胞に豊富に存在し，活性化されたCaMK Ⅱは神経伝達物質合成酵素やシナプス小胞結合蛋白，イオンチャネル，神経伝達物質受容体などのリン酸化を介して，それらの蛋白の機能を調節し，学習・記憶をはじめとする中枢機能に重要な役割を果たしている[17]。生理食塩水連続投与マウスでは，強制水泳試験後に前頭前皮質でCaMK Ⅱのリン酸化レベルが著しく増加するが，PCP連続投与マウスではそのような増加は認められない。CaMK Ⅱ阻害剤であるKN93を強制水泳試験前に前頭前皮質に微量注入すると，CaMK Ⅱリン酸化レベルの低下とともに無動状態の増強が認められる。一方，NMDA受容体のグリシン結合部位に対する部分作動薬であり，NMDA受容体の機能を亢進するD-cycloserineを強制水泳試験前に投与すると，PCP連続投与マウスに認められる無動状態の増強と，CaMK Ⅱリン酸化レベルの低下が改善する。これらの知見から，強制水泳試験においてPCP連続投与マウスで認められる無動状態の増強は，NMDA受容体を介したCaMK Ⅱリン酸化レベルの低下により生じることが示唆される。

以上の知見から，PCP連続投与マウスにみられる強制水泳による無動状態の増強，すなわち陰性症状様の意欲低下は，前頭前皮質におけるグルタミン酸作動性神経系機能の低下に起因することが考えられる。

3．潜在学習障害とグルタミン酸，ドパミン作動性神経系の機能低下

統合失調症患者では，陽性症状や陰性症状ばかりでなく，認知障害も認められる。認知障害として，作業記憶や連合学習の障害が認められるほか，潜在学習の障害も報告されている[18]。水探索試験は，マウスが一度ある環境に曝されたとき，どれだけその環境を認知しているかを調べるもので，装置の構成についての空間的記憶とその中にある給水用のノズルの位置についての記憶を測定することができる。これらの記憶は，自由な探索行動の中でマウスを絶水していなくても獲得されるため，動物の潜在的な学習能力（注意力）を反映すると考えられている。生理食塩水連続投与マウスを絶水させ，再び装置に戻すと，絶水条件で訓練していないのに素早く水を見つけ，訓練を受けていないマウスと比較し，飲水までの時間が短くなる。PCP連続投与マウスでは，訓練した時に給水ノズルへの接触回数などの行動には生理食塩水連続投与マウスと差は認められないが，テスト時にノズルを見つけるまでの時間が延び，潜在学習が障害されている[19]。PCP最終投与から4日間休薬しても，この潜在学習障害は認められる。PCP連続投与による潜在学習障害は，第二世代抗精神病薬であるクロザピンによって緩解されるが，第一世代抗精神病薬であるハロペリドールでは緩解されない[19]。

学習・記憶形成の過程において，NMDA受容体/CaMK Ⅱシグナル経路が重要な役割を果たしていることが知られている[17]。生理食塩水連続投与マウスでは，水探索試験の訓練試行後に前頭前皮質でCaMK Ⅱのリン酸化レベルが著しく増加するが，PCP連続投与マウスではそのような増加は認められない。CaMK Ⅱ阻害剤であるKN93を訓練試行前に前頭前皮質に微量注入すると，潜在学習障害が認められ，前頭前皮質のCaMK Ⅱリ