

## REGULAR RESEARCH ARTICLE

# Role of Prefrontal Serotonergic and Dopaminergic Systems in Encounter-Induced Hyperactivity in Methamphetamine-Sensitized Mice

Tatsunori Tanaka, BSc; Yukio Ago, PhD; Chiaki Umehara, Bsc;  
Emina Imoto, MSc; Shigeru Hasebe, BSc; Hitoshi Hashimoto, PhD;  
Kazuhiro Takuma, PhD; Toshio Matsuda, PhD

Laboratory of Molecular Neuropharmacology (Mr Tanaka, Dr Ago, Ms Umehara, and Dr Hashimoto), and Department of Pharmacology, Graduate School of Dentistry, Osaka University, Osaka, Japan (Mr Hasebe and Dr Takuma); United Graduate School of Child Development, Osaka University, Kanazawa University, Hamamatsu University School of Medicine, Chiba University, and University of Fukui, Osaka, Japan (Drs Hashimoto and Takuma); Division of Bioscience, Institute for Dataability Science (Dr Hashimoto), and Laboratory of Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences (Ms Imoto and Dr Matsuda), Osaka University, Osaka, Japan.

T.T. and Y.A. contributed equally to this work.

Correspondence: Toshio Matsuda, PhD, Laboratory of Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan ([matsuda@phs.osaka-u.ac.jp](mailto:matsuda@phs.osaka-u.ac.jp)).

## Abstract

**Background:** Isolation-reared mice show social encounter-induced hyperactivity with activation of prefrontal serotonergic and dopaminergic systems, but it is not known whether this stress response is observed in other pathological conditions. Here we examined whether the social encounter stimulation induces abnormal behavior during withdrawal in chronic methamphetamine-treated mice.

**Methods:** To induce methamphetamine-induced behavioral sensitization, male mice were injected with methamphetamine (1 mg/kg) once daily for 7 days.

**Results:** The encounter with an intruder elicited hyperactivity 24 h after the last injection of methamphetamine in methamphetamine-sensitized mice. This response was observed even as long as 2 weeks after withdrawal of methamphetamine. The encounter increased c-Fos expression in the prefrontal cortex, dorsal raphe nucleus and ventral tegmental area in methamphetamine-sensitized mice, while it did not in control mice. Furthermore, the encounter increased extracellular serotonin (5-HT) and dopamine, but not noradrenaline, levels in the prefrontal cortex in methamphetamine-sensitized mice. Local injection of 5,7-dihydroxytryptamine and 6-hydroxydopamine into the prefrontal cortex attenuated encounter-induced hyperactivity in methamphetamine-sensitized mice and it markedly decreased prefrontal 5-HT and dopamine levels, respectively. Pharmacological analysis showed that the encounter-induced hyperactivity is mediated by dopamine D<sub>1</sub> receptors and 5-HT<sub>2A</sub> receptors and attenuated by anxiolytics and antidepressants such as diazepam, osetozotan and selective 5-HT reuptake inhibitors. The effect of paroxetine was blocked by the 5-HT<sub>3</sub> receptor antagonist azasetron.

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## Significance Statement

Methamphetamine (METH) is a psychostimulant, and its prolonged use results in dependence or psychosis similar to paranoid schizophrenia. METH withdrawal causes depression and stress-induced anxiety, but the neurochemical mechanism is not fully understood. To study the roles of psychological stress in the expression of abnormal behaviors, we designed an encounter with an intruder to occur through a mesh partition, which avoids physical stress. The present study demonstrates that social encounter stimulation elicits hyperactivity during withdrawal in METH-sensitized mice. Furthermore, we found that the hyperactivity is accompanied by an activation of prefrontal serotonergic and dopaminergic systems and is attenuated by anxiolytic and antidepressant drugs. Our findings imply that encounter-induced behavioral alteration is useful as a novel approach to investigate pharmacotherapy for the symptoms of METH withdrawal.

**Conclusions:** The present study shows that psychological stress elicits hyperactivity with activation of prefrontal 5-HT and dopamine systems in methamphetamine-dependent mice and suggests that the abnormal behavior is associated with anxiety and depression.

**Keywords:** encounter, hyperactivity, methamphetamine, serotonin, dopamine

## Introduction

Aggressive behavior and impaired social interactions are observed in rodent models of psychiatric disorders such as schizophrenia (Sams-Dodd, 1999; Jones et al., 2011; Ago et al., 2014). The response to an encounter with an intruder, a psychological stress, is an example of these abnormal behaviors, and it often involves aggressive contact (i.e., biting attacks, wrestling, and lateral threats) between 2 rodents. To study the involvement of psychological stress in the expression of abnormal behaviors, we designed an encounter stimulation to occur through a mesh partition, which avoids physical stress. We found that the psychological stress elicits a restless and hyperexcitable state (hyperactivity) in isolation-reared mice, a model of psychiatric disorders, and it specifically activates prefrontal dopaminergic and serotonergic systems (Ago et al., 2013; Araki et al., 2014). Furthermore, we showed that the hyperactivity is attenuated by the anxiolytic diazepam and several antidepressants (Ago et al., 2013; Hasebe et al., 2015). The pharmacological profile of encounter-induced hyperactivity in isolation-reared mice suggests that the hyperactivity reflects an anxiety- and depression-like state. It is not known whether the encounter-induced abnormal behavior is observed in other rodent models of psychiatric disorders.

Methamphetamine (METH) is a psychostimulant, and its prolonged use results in dependence or psychosis similar to paranoid schizophrenia (Pierce and Kalivas, 1997). In rodents, chronic METH administration causes behavioral sensitization, a long-lasting augmented locomotor response (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Ago et al., 2008). This behavioral sensitization is used as an animal model of METH dependence and psychosis, and the mesocorticolimbic dopamine and serotonin (5-HT) neurons play key roles as the neural substrates (Vanderschuren and Kalivas, 2000; Nestler, 2001; Ago et al., 2008). METH withdrawal causes sedation, depression, and stress-induced anxiety (London et al., 2004; Mancino et al., 2011; Shen et al., 2013; Hellem, 2016), and central dopamine and 5-HT systems as well as  $\gamma$ -aminobutyric acid (GABA) and glutamate systems play an important role in anxiety disorders (Kent et al., 2002; Nikolaus et al., 2010) and psychostimulant withdrawal and relapse (Ago et al., 2008; Collo et al., 2014; Neisewander et al., 2014; Filip et al., 2015; Li et al., 2015). These observations suggest that METH-dependent rodents should show abnormal behavior in response to encounter stimulation, a psychological

stress. In contrast, Janetsian et al. (2015) reported that deficits in social interaction were not observed during withdrawal in chronic METH-treated mice. There is little information on the effect of psychological stress on behavior in psychostimulant-dependent rodents. The present study demonstrates that psychological stress elicits hyperactivity during withdrawal in METH-sensitized mice. Furthermore, we found that the prefrontal serotonergic and dopaminergic systems play a key role in the encounter-induced hyperactivity in METH-sensitized mice.

## Materials and Methods

### Animals and Drugs

All animal studies were approved by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University. All experimental procedures were conducted in accordance with the guidelines of the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996). Every effort was made to minimize animal suffering and to reduce the number of animals used. Seven-week-old male ddY mice were obtained from SHIMIZU Laboratory Supplies Co., Ltd. (Kyoto, Japan) and housed in cages (28 cm  $\times$  17 cm  $\times$  12 cm) in groups of 5 or 6 animals under controlled environmental conditions (22  $\pm$  1°C; 50  $\pm$  10% relative humidity; 12-hour-light/dark cycle, lights on at 8:00 AM; food and water ad libitum) for at least 1 week before use in the experiments. The following drugs were used: methamphetamine hydrochloride (METH; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), MDL100907, desipramine hydrochloride, diazepam, escitalopram oxalate, fluvoxamine maleate, paroxetine maleate, ritanserin, 5,7-dihydroxytryptamine (5,7-DHT) creatinine sulfate, 6-hydroxydopamine hydrobromide (6-OHDA) (Sigma, St. Louis, MO), GR125487 sulfamate, SB399885 hydrochloride, SB269970 hydrochloride, RS102221 hydrochloride hydrate, SCH39166 hydrobromide, raclopride, SR57227 hydrochloride (Tocris Bioscience, Bristol, UK), azasetron hydrochloride, osemozotan, WAY100635 (Mitsubishi Tanabe Pharma Corp., Yokohama, Japan).

METH, azasetron, desipramine, escitalopram, fluvoxamine, GR125487, paroxetine, SB399885, and WAY100635 were dissolved in saline (0.9% wt/v NaCl solution). Diazepam, osemozotan, and ritanserin were suspended in 0.5% wt/v carboxymethylcellulose.

MDL100907, raclopride, and SCH39166 were dissolved in saline containing <0.01% v/v dimethyl sulfoxide. RS102221 was first dissolved in sterile distilled water containing 0.1 M HCl, and the pH was adjusted to 7.4 using 0.1 M NaOH. 5,7-DHT and 6-OHDA were dissolved in Ringer's solution (147.2 mM NaCl, 4.0 mM KCl, and 2.2 mM CaCl<sub>2</sub> [pH 6.0]; Fuso Pharmaceutical Industries, Ltd., Osaka, Japan) containing 0.1% w/v ascorbic acid. All drugs except 5,7-DHT and 6-OHDA were administered i.p. at a volume of 10 mL/kg body weight. In this study, a single dose of most of the drugs was used. Thus, the dosage and timing of drugs used here were determined referring to our and others' previous studies where the drugs affected behaviors and/or neurotransmitter release (Moser et al., 1996; Ago et al., 2006a, 2006b, 2007, 2011b, 2013, 2015; Collins et al., 2010; Jones et al., 2010; Hiramatsu et al., 2013; Bétry et al., 2015; Hasebe et al., 2015). To induce METH-induced behavioral sensitization, mice were injected with METH (1 mg/kg) once daily for 7 days, as previously described (Ago et al., 2006b, 2007, 2012). Mice withdrawn for 7 days after chronic METH or saline treatment were used for the experiments unless otherwise stated.

### Social Encounter Stimulation and Behavioral Analysis

The social encounter stimulations and behavioral analyses were performed as previously described (Ago et al., 2013; Araki et al., 2014; Hasebe et al., 2015). A METH- or saline-pretreated male mouse was placed in the large compartment of a novel clear Plexiglas cuboid cage (30 × 30 × 35 cm), which was divided into 2 compartments by a mesh partition (Figure 1A). This allowed the animal to see, hear, and smell, but not physically contact, the neighbor. After a 3-hour habituation period, a METH- or saline-pretreated male mouse (the resident) was injected with the drug or vehicle. An unfamiliar age-matched male ddY mouse was introduced into the unoccupied smaller compartment as an intruder 30 minutes after the drug administration. The resident and intruder mice were allowed to interact through the partition for 20 minutes before the intruder mouse was removed. The behavior of the resident mouse during the 20-minute encounter was videotaped, and its locomotor path and distance travelled in the large compartment were automatically analyzed offline using the ANY-maze video tracking software (Stoelting Company, Wood Dale, IL). The locomotor activity of the resident mouse in areas near and far from the partition was analyzed (Figure 1B), and the distance travelled near the partition was used as an index of the encounter response.

### In Vivo Microdialysis

Microdialysis experiments were performed as previously described (Ago et al., 2011b; Hiramatsu et al., 2013; Hara et al., 2016). Briefly, each mouse was anesthetized with a mixture of medetomidine (0.3 mg/kg, i.p.), midazolam (4 mg/kg, i.p.), and butorphanol (5 mg/kg, i.p.) and stereotaxically implanted unilaterally and counterbalanced left or right with a guide-cannula for a dialysis probe (Eicom Corp., Kyoto, Japan) in the prefrontal cortex (A +1.9 mm, L +/-0.5 mm, V -0.8 mm, from bregma and skull; Franklin and Paxinos, 1997). The cannula was cemented in place with dental acrylic, and the animal was kept warm and allowed to recover from anesthesia. Postoperative analgesia was performed with a single injection of buprenorphine (0.1 mg/kg, i.p.). Two days after surgery, the probe was perfused with Ringer's solution at a constant flow rate of 1 µL/min. A stabilization period of 3 hours, which was identical to the 3-hour habituation

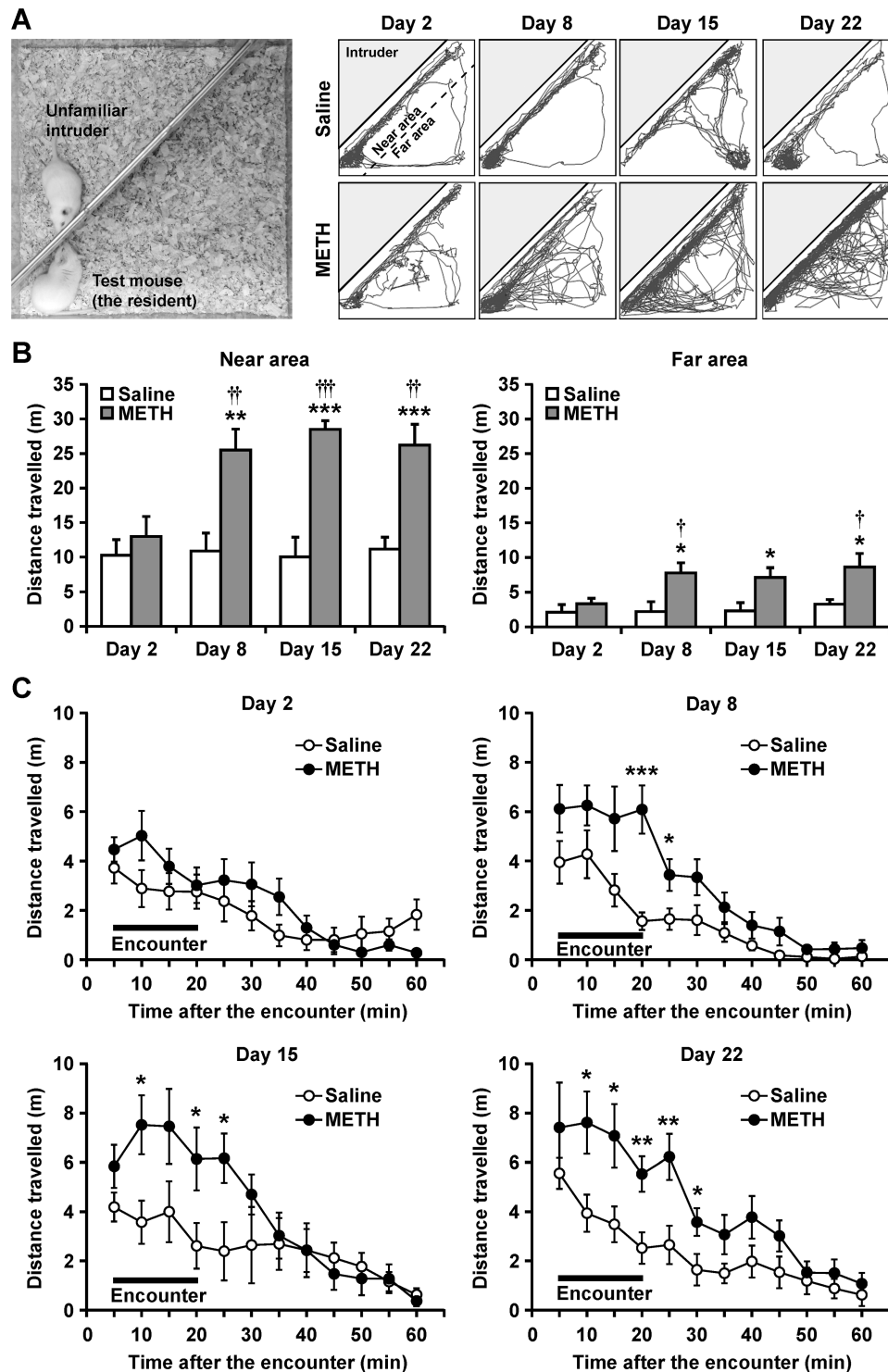
period in the clear Plexiglas cage described above, was established before the onset of the experiment. Microdialysis samples (20 µL) were collected every 20 minutes and injected immediately onto a high-performance liquid chromatography column for simultaneous assaying of 5-HT, dopamine, and noradrenaline (Ago et al., 2011b; Hiramatsu et al., 2013; Hara et al., 2016).

### 5,7-DHT and 6-OHDA Treatment

Intracerebral drug administration was performed as previously described (Ago et al., 2011a). Twenty-four hours after repeated METH administration, mice were pretreated with desipramine (15 mg/kg, i.p.) 30 minutes prior to surgery to prevent the destruction of noradrenergic neurons by 5,7-DHT. For 6-OHDA infusion, mice were pretreated with desipramine (35 mg/kg, i.p.) and escitalopram (5 mg/kg, i.p.) 30 minutes prior to surgery to prevent the destruction of noradrenergic and serotonergic neurons (Bergamini et al., 2016). Mice were subsequently anesthetized with a mixture of medetomidine, midazolam, and butorphanol. Either 5,7-DHT or 6-OHDA was delivered bilaterally into the prefrontal cortex (stereotaxic coordinates: A +1.9 mm, L ±0.5 mm, V -2.5 mm, from bregma and skull; Franklin and Paxinos, 1997) using a 28-gauge stainless-steel injector connected to a microsyringe pump. A volume of 1 µL of 5,7-DHT (6 µg/µL) or 6-OHDA (1.5 µg/µL) was infused over a period of 10 minutes on each side. After infusion, the cannula was left in place for a further 5 minutes to allow diffusion of the neurotoxin and to avoid backflow of the solution up the injection path. Sham-operated mice underwent the same surgical procedure and received an equal volume of vehicle solution. Mice received an injection of buprenorphine (0.1 mg/kg, i.p.) and gentamicin (10 mg/kg, i.p.) and recovered within a few hours after the surgery. The mice were used for experiments 1 week after the surgery. Tissue levels of 5-HT, dopamine, and noradrenaline in the prefrontal cortex and striatum were determined as described previously (Kawasaki et al., 2006; Horiguchi et al., 2013; Ota et al., 2015).

### c-Fos Immunohistochemistry

Brain expression of the neuronal activity marker c-Fos was determined 2 hours after the 20-minute encounter with an intruder. Control mice were placed into a chamber with no neighbor. c-Fos immunostaining was performed as described previously (Ago et al., 2013, 2015). Each mouse was deeply anesthetized with isoflurane and perfused transcardially with saline, followed by a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS). Serial 20-µm-thick coronal sections were cut using a cryostat microtome at -20°C. Free-floating sections were pre-incubated for 30 minutes in 0.3% hydrogen peroxide in PBS and then blocked in 1.5% goat serum in PBS for 20 minutes at room temperature. Thereafter, the sections were incubated with anti-c-Fos rabbit polyclonal primary antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature. Subsequently, the sections were washed in PBS and incubated with a secondary antibody solution containing biotinylated anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The sections were then incubated with avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 minutes at room temperature. Brown cytosolic products were obtained by reacting with 3,3'-diaminobenzidine (Sigma, St. Louis, MO). Four independent sections per animal containing the prefrontal cortex, striatum, nucleus accumbens, dorsal raphe nucleus, ventral tegmental area, locus coeruleus, paraventricular hypothalamic



**Figure 1.** Effects of social encounter stimulation on the behavior of mice. (A) Experimental apparatus and representative locomotor paths of resident methamphetamine (METH)- or saline-treated mice during the 20-minute encounters. At 24 hours after a single administration of METH (1 mg/kg) or saline, each mouse was subjected to the encounter with the intruder (on day 2). Then, mice were administered METH (1 mg/kg/d) or saline once daily until day 7. At 24 hours and 8 and 15 days after the last METH injection, each mouse was subjected to the encounter with the intruder (on days 8, 15, and 22, respectively). (B) Total distance travelled during the 20-minute encounters in areas either near or far from the partition was analyzed. Results are expressed as the mean  $\pm$  SEM of 12 mice/group. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with the saline-treated mice on each day. † $P < .05$ , †† $P < .01$ , ††† $P < .001$ , compared with the METH-treated mice on day 2. (C) Time-course of the distance travelled in the area near the partition during and after the 20-minute encounters was shown. Results are expressed as the mean  $\pm$  SEM of 12 mice/group. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with the saline-treated mice on each time point.

nucleus, or central or basolateral nucleus of the amygdala were selected. c-Fos-positive cells were counted manually by experienced observers blinded to treatment and encounter conditions

under bright-field illumination on an Axio Imager.M2 microscope (Carl Zeiss, Jena, Germany). The number of c-Fos-positive cells in each section was determined in a 500- $\mu\text{m} \times 500\text{-}\mu\text{m}$  area



in the left and right hemispheres and averaged using the ImageJ 1.41 software package (NIH, Bethesda, MD). The average of this average across 4 sections was then calculated for each subject.

### Statistical Analysis

All results are presented as the mean  $\pm$  SEM. For the microdialysis experiment, data were calculated as percentage change from baseline dialysate concentrations, with 100% defined as the average of three measurements before the encounter. Data for the time-course of the encounter responses (Figure 1) and microdialysis (Figure 2) were analyzed using 2-way ANOVA with treatment as the intersubject factor and repeated measures with time as the intrasubject factor, followed by the Bonferroni/Dunn posthoc test. For the other experiments, data were analyzed using 2-way ANOVA with the treatment and/or encounter as the independent variables, followed by the Bonferroni/Dunn posthoc test. Statistical analyses were performed using the software package Statview 5.0J for Apple Macintosh (SAS Institute Inc., Cary, NC). Values of  $P < .05$  were considered statistically significant.

## Results

### Effects of Social Encounter Stimulation on the Behavior of METH-Sensitized Mice

Figure 1 shows the effects of social encounter stimulation on the behavior of chronic METH-treated (METH-sensitized) and saline-treated (control) mice. Spontaneous locomotor activity in the cage decreased gradually during the 3-hour habituation period, and there was no difference in activity between chronic METH- and saline-treated mice during this time (data not shown). Figure 1A shows the representative locomotor paths of resident METH- and saline-treated mice during the 20-minute encounters. Both groups of mice moved around in their area, especially near the partition, and interacted with the intruder over the partition, suggesting behavioral reactivity to the intruder. At 24 hours after a single administration of METH (1 mg/kg) or saline (on day 2), locomotor activities of METH-treated mice in near or far area did not differ from those of saline-treated mice (Figure 1B). At 24 hours after the repeated administration of METH (1 mg/kg/d, once daily for 7 days) (on day 8), locomotor activities of METH-sensitized mice in the area near the partition were significantly greater

than those of saline-treated control mice, suggesting hyperactivity in response to the intruder. This encounter-induced hyperactivity in the near area was also observed after a 7-day or 14-day withdrawal period (on days 15 and 22, respectively). Repeated measures 2-way ANOVA revealed significant main effects for METH treatment ( $F_{1,22} = 39.0, P < .0001$ ) and time ( $F_{3,66} = 4.4, P < .01$ ), and there was a significant interaction between treatment and time ( $F_{3,66} = 4.1, P < .01$ ). The temporal pattern of locomotor activity of METH-sensitized mice in the area far from the partition was similar to that in the near area, but repeated-measures 2-way ANOVA indicated no significant interaction between treatment and time ( $F_{3,66} = 1.2, P > .05$ ). Because of this small difference between areas, we used the distance travelled near the partition as an index of the encounter-induced behavioral response for the subsequent analyses. Figure 1C shows the time-course of the locomotor response in the area near the partition during and after the 20-minute encounters. Enhanced locomotor activity in METH-sensitized mice was still observed for several minutes following the cessation of the encounter on day 8 (interaction:  $F_{11,242} = 2.7, P < .01$ ), 15 (interaction:  $F_{11,242} = 3.3, P < .001$ ), and 22 (treatment:  $F_{1,22} = 14.8, P < .001$ ).

### Effects of Social Encounter Stimulation on Extracellular Monoamine Levels in the Prefrontal Cortex and Regional Neuronal Activity in the Brain

Figure 2 shows the effects of social encounter stimulation on extracellular levels of monoamines in the prefrontal cortex of mice. Baseline levels of extracellular 5-HT, dopamine and noradrenaline (not corrected for in vitro probe recovery) in the prefrontal cortex did not differ significantly between chronic METH- and saline-treated mice (analyzed using Student's *t* test). The 5-HT levels (mean  $\pm$  SEM) were  $1.14 \pm 0.22$  (METH,  $n = 8$ ) and  $1.01 \pm 0.17$  (saline,  $n = 8$ ) pg/20  $\mu$ L. The dopamine levels were  $0.68 \pm 0.10$  (METH) and  $0.51 \pm 0.09$  (saline) pg/20  $\mu$ L. The noradrenaline levels were  $0.80 \pm 0.24$  (METH) and  $0.78 \pm 0.05$  (saline) pg/20  $\mu$ L. Encounter stimulation caused significant increases in extracellular 5-HT and dopamine levels, but not noradrenaline levels, in the prefrontal cortex of chronic METH-treated mice. In contrast, the stimulation did not affect extracellular monoamine levels in the prefrontal cortex of saline-treated mice (interaction between time and treatment:  $F_{8,112} = 3.0, P < .01$  for 5-HT;  $F_{8,112} = 2.2, P < .05$  for dopamine;  $F_{8,112} = 0.7, P > .05$  for noradrenaline).

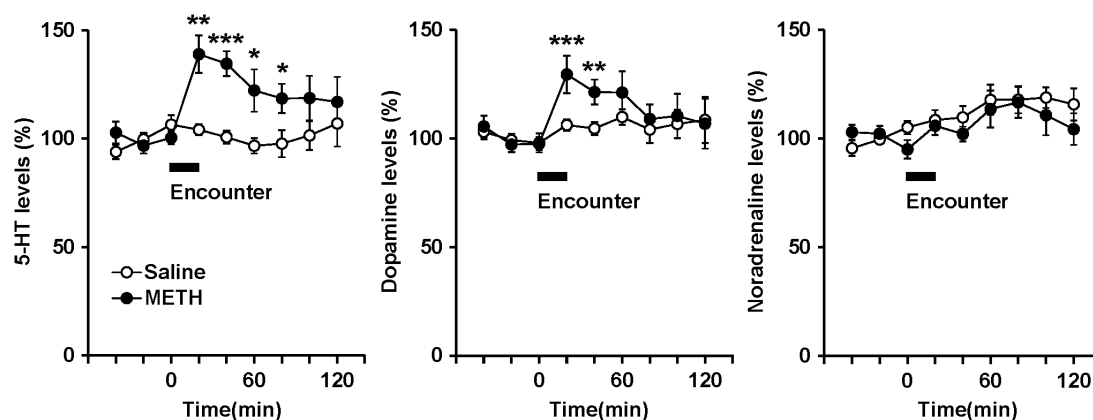


Figure 2. Effects of social encounter stimulation on extracellular monoamine levels of mice. Mice were administered methamphetamine (METH; 1 mg/kg) or saline repeatedly for 7 days. At 7 days after the withdrawal period, each mouse was subjected to the 20-minute encounter with the intruder, as indicated by the horizontal bar. Results are expressed as the mean  $\pm$  SEM of 8 mice/group. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with saline-pretreated mice at each time point.

Expression of the neuronal activity marker c-Fos was determined 2 hours after the social encounters in the prefrontal cortex, striatum, nucleus accumbens, dorsal raphe nucleus, ventral tegmental area, and locus coeruleus of mice (Figures 3 and 4). Representative photomicrographs of c-Fos-positive cells are shown in Figure 3. Encounter stimulation caused a significant increase in the number of c-Fos-positive nuclei in the prefrontal cortex (main effect of the encounter:  $F_{1,36} = 30.4$ ,  $P < .0001$ ; interaction between the encounter and treatment:  $F_{1,36} = 18.1$ ,  $P < .001$ ), dorsal raphe nucleus (main effect:  $F_{1,36} = 21.7$ ,  $P < .0001$ ; interaction:  $F_{1,36} = 4.2$ ,  $P < .05$ ), and ventral tegmental area (main effect:  $F_{1,36} = 13.2$ ,  $P < .001$ ; interaction:  $F_{1,36} = 4.4$ ,  $P < .05$ ) of chronic METH-treated mice, but not saline-treated mice (Figure 4). Encounter stimulation increased c-Fos expression in the striatum (main effect of the encounter:  $F_{1,36} = 9.3$ ,  $P < .01$ ) and nucleus accumbens shell (main effect:  $F_{1,36} = 15.8$ ,  $P < .001$ ) of both chronic METH- and saline-treated mice, but there was no significant interaction between the encounter and METH treatment. Encounter stimulation did not affect c-Fos expression in the locus coeruleus (Figure 4F), nucleus accumbens core, paraventricular hypothalamic nucleus, or the central or basolateral nucleus of the amygdala (data not shown).

### Effects of Prefrontal 5-HT and Dopamine Depletion on Encounter-Induced Hyperactivity in METH-Sensitized Mice

To clarify the roles of the prefrontal serotonergic and dopaminergic systems, we examined the effects of bilateral microinjection of either the serotonergic toxin 5,7-DHT, or dopaminergic toxin 6-OHDA into the prefrontal cortex on encounter-induced hyperactivity in METH-sensitized mice (Figure 5). Local injection of 5,7-DHT (6  $\mu\text{g}/\text{side}$ ) induced marked reductions in

concentrations of 5-HT, but not dopamine or noradrenaline, in the prefrontal cortex of both chronic METH- and saline-treated mice (Table 1). This treatment did not affect the monoamine levels in the striatum (Table 1). Prefrontal 5-HT depletion by 5,7-DHT abrogated encounter-induced hyperactivity in chronic METH-treated mice (Figure 5A) (interaction between 5,7-DHT and METH treatments:  $F_{1,44} = 7.1$ ,  $P < .05$ ), while it did not affect the response to the encounter in saline-treated mice. Local injection of 6-OHDA (1.5  $\mu\text{g}/\text{side}$ ) induced marked reductions in concentrations of dopamine, but not 5-HT or noradrenaline, in the prefrontal cortex of both chronic METH- and saline-treated mice (Table 2). This treatment did not affect the monoamine levels in the striatum (Table 2). Prefrontal dopamine depletion by 6-OHDA abrogated encounter-induced hyperactivity in chronic METH-treated mice (Figure 5B) (interaction between 6-OHDA and METH treatments:  $F_{1,44} = 4.4$ ,  $P < .05$ ), while it did not affect the response to the encounter in saline-treated mice.

### Involvement of 5-HT and Dopamine Receptors in Encounter-Induced Hyperactivity in METH-Sensitized Mice

To investigate which subtypes of 5-HT and/or dopamine receptors are involved in the encounter-induced hyperactivity, we examined the effects of antagonists of various 5-HT or dopamine receptor subtypes on the encounter-induced hyperactivity in METH-sensitized mice (Figure 6). Ritanserin (3 mg/kg), a nonselective 5-HT<sub>2</sub> receptor antagonist, blocked the encounter-induced hyperactivity in chronic METH-treated mice (interaction between the METH and antagonist treatments:  $F_{6,154} = 2.2$ ,  $P < .05$ ) (Figure 6A). None of the other 5-HT receptor antagonists, 5-HT<sub>1A</sub> receptor antagonist (WAY100635, 1 mg/kg), 5-HT<sub>3</sub> receptor antagonist (azasetron, 3 mg/kg), 5-HT<sub>4</sub> receptor antagonist (GR125487, 3 mg/kg), 5-HT<sub>6</sub> receptor antagonist (SB399885, 3 mg/kg) or 5-HT<sub>7</sub> receptor antagonist (SB269970, 1 mg/kg) affected the hyperactivity. The 5-HT<sub>2A</sub> receptor antagonist MDL100907 (1 mg/kg), but not the 5-HT<sub>2C</sub> receptor antagonist RS102221 (1 mg/kg), inhibited the encounter-induced hyperactivity in chronic METH-treated mice ( $F_{2,66} = 3.7$ ,  $P < .05$ ) (Figure 6B). Furthermore, the dopamine-D<sub>1/5</sub> receptor antagonist SCH39166 (0.2 mg/kg), but not the dopamine-D<sub>2/3</sub> receptor antagonist raclopride (0.1 mg/kg), inhibited the encounter-induced hyperactivity in chronic METH-treated mice ( $F_{2,66} = 3.2$ ,  $P < .05$ ) (Figure 6C).

### Effects of Anxiolytics and Antidepressants on Encounter-Induced Hyperactivity in METH-Sensitized Mice

We previously found that encounter-induced hyperactivity in isolation-reared mice is attenuated by the anxiolytic diazepam and several antidepressants (Ago et al., 2013; Hasebe et al., 2015). In addition, the 5-HT<sub>1A</sub> receptor agonist osemozotan, which shows anxiolytic activities in rodent models (Sakaue et al., 2001, 2003; Matsuda, 2013; Ago et al., 2014), also inhibited encounter-induced hyperactivity in isolation-reared mice (Ago et al., 2013). To investigate the pharmacological profile of encounter-induced hyperactivity in METH-sensitized mice, we examined the effects of the GABA<sub>A</sub> receptor agonist diazepam (1 mg/kg), the 5-HT<sub>1A</sub> receptor agonist osemozotan (0.3 mg/kg), and the selective 5-HT reuptake inhibitors (SSRIs) paroxetine (10 mg/kg) and fluvoxamine (30 mg/kg) on encounter-induced hyperactivity (Figure 7). All drugs tested abrogated the encounter-induced hyperactivity in chronic METH-treated mice (interaction between the treatments:  $F_{1,44} = 5.8$ ,  $P < .05$  for diazepam;  $F_{1,44} = 6.2$ ,  $P < .05$  for osemozotan;  $F_{1,44} = 6.8$ ,

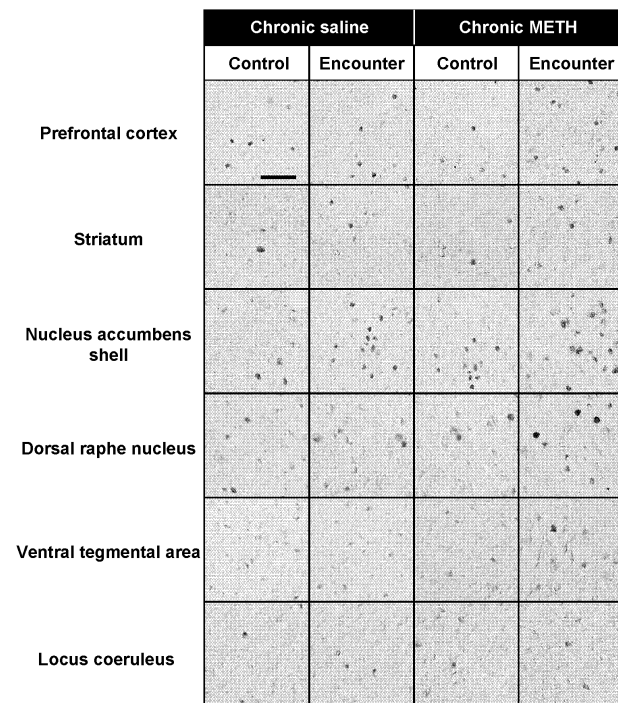


Figure 3. Representative photomicrographs showing c-Fos-immunoreactive cells after encounter with an intruder in the brain of resident test mice. Mice that were not exposed to an intruder were used as controls (Control). Scale bar = 50  $\mu\text{m}$ .

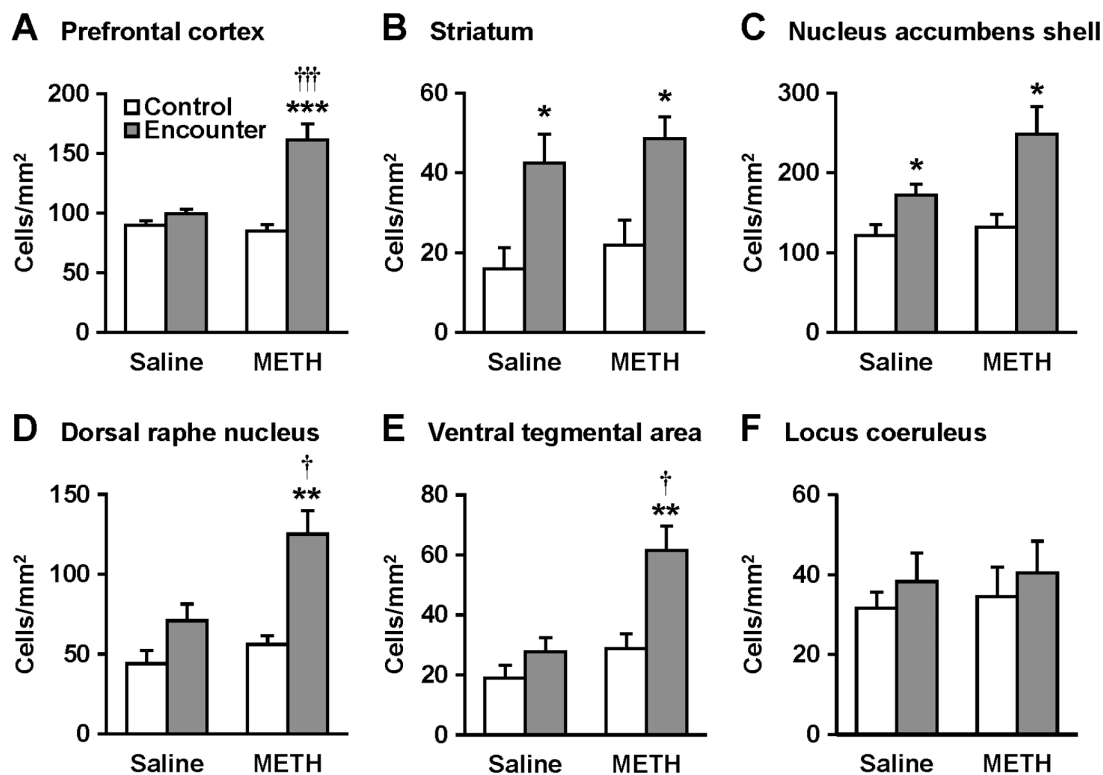


Figure 4. Effects of social encounter stimulation on c-Fos expression in the prefrontal cortex (A), striatum (B), nucleus accumbens shell (C), dorsal raphe nucleus (D), ventral tegmental area (E), and locus coeruleus (F) of mice. Immunohistochemical localization of the neuronal activity marker c-Fos in the chronic methamphetamine (METH)- and saline-pretreated mice was determined 2 hours after the 20-minute encounter. Mice that were not exposed to an intruder were used as controls. Results are expressed as the mean  $\pm$  SEM of 10 mice/group. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with the control in each group. † $P < .05$ , †† $P < .001$ , compared with saline-pretreated mice.

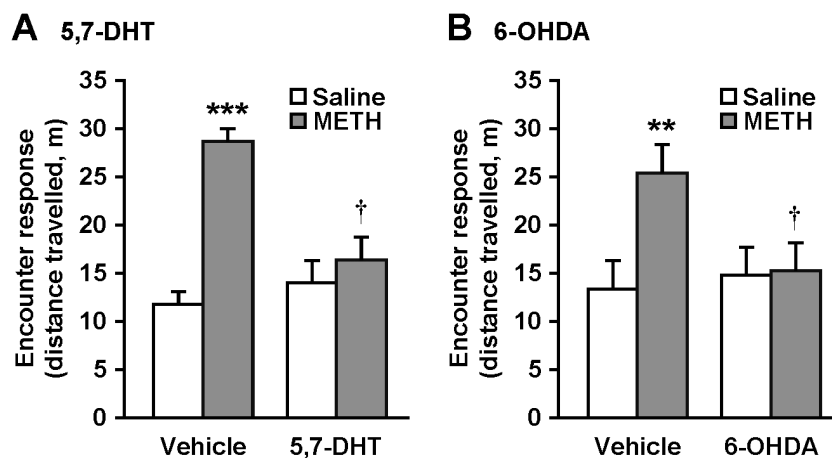


Figure 5. Effects of local injection of either 5,7-DHT or 6-OHDA into the prefrontal cortex on encounter-induced hyperactivity in methamphetamine (METH)-sensitized mice. Twenty-four hours after repeated METH or saline administration, mice received bilateral microinjection of 5,7-DHT (6  $\mu$ g/side) (A), 6-OHDA (1.5  $\mu$ g/side) (B), or vehicle into the prefrontal cortex. At 7 days after surgery, each mouse was subjected to the encounter with an intruder. Total distance travelled in the area near the partition during the 20-minute encounters was analyzed as an index of the encounter response. Results are expressed as the mean  $\pm$  SEM of 12 mice/group. \*\* $P < .01$ , \*\*\* $P < .001$ , compared with vehicle-injected saline-pretreated mice. † $P < .05$ , compared with vehicle-injected mice in each group.

$P < .05$  for paroxetine;  $F_{1,44} = 5.7$ ,  $P < .05$  for fluvoxamine), while they did not affect the encounter response in saline-treated mice.

#### Role of the 5-HT<sub>3</sub> Receptor in the Inhibitory Effect of Paroxetine on Encounter-Induced Hyperactivity

Finally, we examined which subtypes of 5-HT receptors are involved in the effect of paroxetine (Figure 8). The 5-HT<sub>3</sub> receptor

antagonist azasetron blocked the inhibitory effect of paroxetine on the encounter-induced hyperactivity in chronic METH-treated mice ( $F_{6,77} = 7.3$ ,  $P < .0001$ ), while the other 5-HT receptor antagonists used here did not impact the effect of paroxetine (Figure 8A). In support of the involvement of the 5-HT<sub>3</sub> receptor, the 5-HT<sub>3</sub> receptor agonist SR57227 (0.3 and 1 mg/kg) attenuated the encounter-induced hyperactivity in METH-sensitized mice ( $F_{2,66} = 6.3$ ,  $P < .01$ ) (Figure 8B).

**Table 1.** Effects of 5,7-DHT on Contents of Monoamines in the Prefrontal Cortex and Striatum of Mice

	Chronic Saline		Chronic METH	
	Vehicle	5,7-DHT	Vehicle	5,7-DHT
<b>Prefrontal cortex</b>				
5-HT	681 ± 62	75 ± 16***	759 ± 99	100 ± 39***
Dopamine	33 ± 7	29 ± 4	37 ± 2	28 ± 3
Noradrenaline	597 ± 38	490 ± 48	665 ± 49	515 ± 66
<b>Striatum</b>				
5-HT	396 ± 46	508 ± 103	342 ± 56	415 ± 59
Dopamine	8782 ± 1412	10871 ± 1625	9927 ± 1753	10260 ± 1924
Noradrenaline	76 ± 7	95 ± 17	86 ± 7	88 ± 6

Results are expressed as the mean ± SEM of 11–12 mice/group. Values are expressed as ng/g tissue (wet weight). \*\*\*P < .001, compared with vehicle-treated mice.

**Table 2.** Effects of 6-OHDA on Contents of Monoamines in the Prefrontal Cortex and Striatum of Mice

	Chronic Saline		Chronic METH	
	Vehicle	6-OHDA	Vehicle	6-OHDA
<b>Prefrontal Cortex</b>				
5-HT	706 ± 69	691 ± 69	699 ± 41	681 ± 76
Dopamine	52 ± 7	16 ± 2***	46 ± 5	16 ± 2***
Noradrenaline	397 ± 39	347 ± 40	417 ± 38	367 ± 41
<b>Striatum</b>				
5-HT	369 ± 31	375 ± 22	354 ± 27	335 ± 12
Dopamine	13445 ± 638	12401 ± 783	12892 ± 1049	12992 ± 902
Noradrenaline	80 ± 8	72 ± 7	79 ± 8	72 ± 8

Results are expressed as the mean ± SEM of 12 mice/group. Values are expressed as ng/g tissue (wet weight). \*\*\*P < .001, compared with vehicle-treated mice.

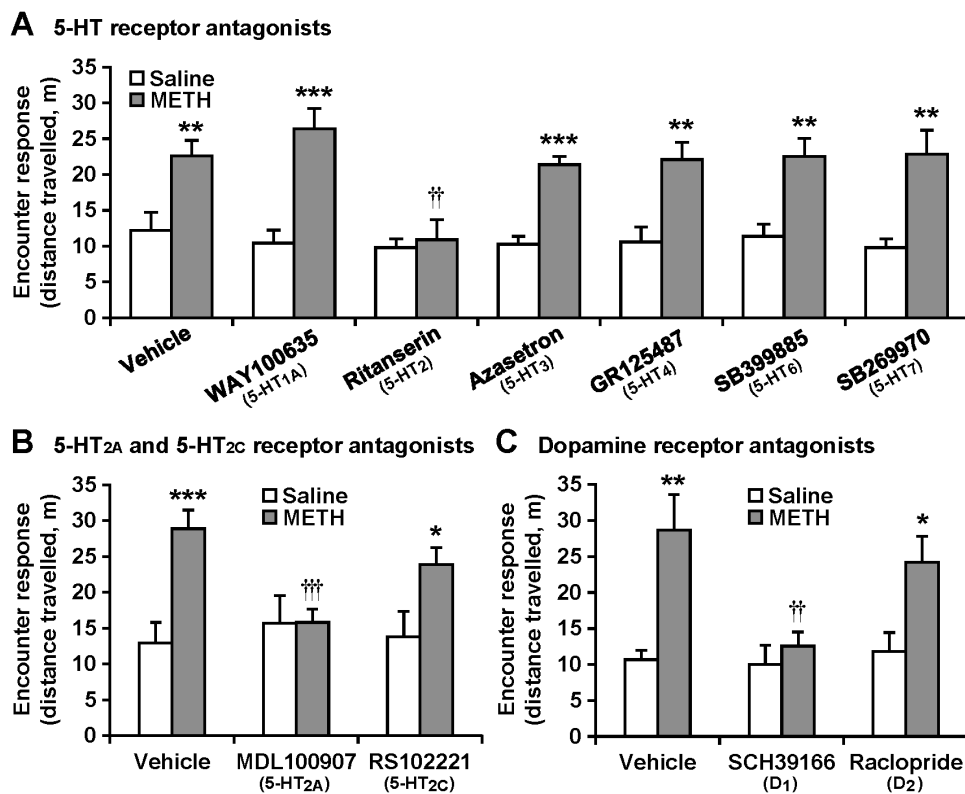
## Discussion

Using a cage divided into 2 compartments by a mesh partition to prevent direct physical interactions, we have found that an encounter with an intruder elicits hyperactivity in isolation-reared mice (Ago et al., 2013). Although the exact profile of the encounter-induced response is not known, previous pharmacological studies suggested that the behavior is associated with anxiety and depression, because it is attenuated by diazepam and antidepressants (Ago et al., 2013; Hasebe et al., 2015). However, it is not known whether the encounter-induced hyperactivity is observed in other models of psychiatric disorders. As withdrawal from psychostimulant use is accompanied by symptoms of anxiety and depression in both humans and animal models (Barr and Markou, 2005; Kitanaka et al., 2008; Barr et al., 2010; Vuong et al., 2010), it is possible that mice chronically treated with psychostimulants show encounter-induced hyperactivity. Building on our previous studies on METH-sensitized mice (Ago et al., 2006b, 2007, 2012), the present study examined the effect of encounter stimulation on behavior in METH-sensitized mice. The present study demonstrated that a psychological stress elicits hyperactivity during withdrawal in chronic, but not acute, METH-treated mice. The hyperactivity was observed at both 24 hours and 2 weeks after the last injection of METH, and the time course of the hyperactivity was similar to expression of behavioral sensitization as reported previously (Ago et al., 2006b, 2007, 2012). Therefore, it appears that the encounter stimulation-induced hyperactivity observed during withdrawal in METH-sensitized mice is a phenotype leading to relapse.

Concerning the mechanism for the encounter-induced hyperactivity in METH-sensitized mice, the present study examined the effect of the encounter stimulation on prefrontal monoamine levels and c-Fos expression. The encounter increased prefrontal 5-HT and dopamine, but not noradrenaline,

release. Furthermore, it increased c-Fos expression in the prefrontal cortex, dorsal raphe, and ventral tegmental area. These results suggest that prefrontal serotonergic and dopaminergic systems play a role in encounter-induced hyperactivity, as reported in isolation-reared mice (Ago et al., 2013). We also found that microinjection of either 5,7-DHT or 6-OHDA into the prefrontal cortex blocked the encounter-induced hyperactivity. Treatment with 5,7-DHT and 6-OHDA selectively decreases the 5-HT and dopamine levels, respectively, in the prefrontal cortex. This result therefore supports an important role for the prefrontal serotonergic and dopaminergic systems in encounter-induced hyperactivity. Furthermore, the hyperactivity was attenuated by the 5-HT<sub>2</sub> receptor antagonist ritanserin, the 5-HT<sub>2A</sub> receptor antagonist MDL100907, and the dopamine-D<sub>1</sub> receptor antagonist SCH39166, but not by other 5-HT receptor subtype antagonists and the dopamine-D<sub>2</sub> receptor antagonist raclopride. These results suggest that 5-HT<sub>2A</sub> and dopamine-D<sub>1</sub> receptors are involved in the hyperactivity in METH-sensitized mice. Furthermore, the observation that MDL100907 and SCH39166 show a complete blockade of the encounter-induced hyperactivity suggests that simultaneous activation of 5-HT<sub>2A</sub> and dopamine-D<sub>1</sub> receptors plays a key role in the expression of the abnormal behavior in METH-sensitized mice. It is not known how the activation of these receptors results in the expression of the abnormal behavior in METH-sensitized mice. With respect to the effects of ritanserin, MDL100907, and osetozotan, Martin-Ruiz et al. (2001) demonstrated that 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptors are colocalized in cortical pyramidal neurons and they have opposing regulatory effects on prefrontal serotonergic transmission. Therefore, the possibility remains that the effects of ritanserin, MDL100907, and osetozotan are owing to inhibition of prefrontal 5-HT release.





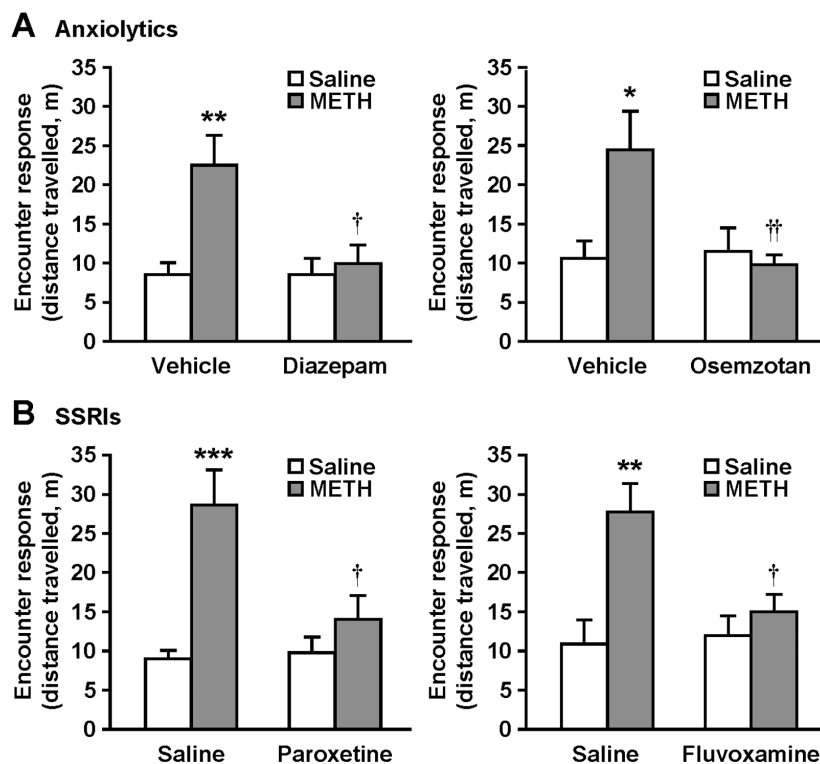
**Figure 6.** Effects of serotonin (5-HT) and dopamine receptor antagonists on encounter-induced hyperactivity in methamphetamine (METH)-sensitized mice. The resident chronic METH- or saline-pretreated mice were injected (i.p.) with the 5-HT receptor subtype antagonists WAY100635 (1 mg/kg), ritanserin (3 mg/kg), azasetron (3 mg/kg), GR125487 (3 mg/kg), SB399885 (3 mg/kg), or SB269970 (1 mg/kg) (A), MDL100907 (1 mg/kg) or RS102221 (1 mg/kg) (B), the dopamine receptor antagonists SCH39166 (0.2 mg/kg) or raclopride (0.1 mg/kg) (C), or vehicle 30 minutes before the encounter with the intruder. Total distance travelled in the area near the partition during the 20-minute encounters was analyzed as an index of the encounter response. Results are expressed as the mean  $\pm$  SEM of 12 mice/group. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with saline-pretreated mice in each antagonist-treated group. † $P < .01$ , †† $P < .001$ , compared with vehicle-injected METH-pretreated mice.

In this study, neurotoxic lesions and any 5-HT and dopamine receptor antagonists did not alter the locomotor response to the encounter in saline-pretreated mice. The effects of 6-OHDA and 5,7-DHT microinjection into the prefrontal cortex on locomotor activity were similar to the previous studies (Li et al., 2010; Sziray et al., 2010; Wanchoo et al., 2010). Furthermore, the receptor antagonists did not affect the baseline locomotor activity, although Collins et al. (2010) reported that the dopamine-D<sub>1</sub> receptor antagonist SCH39166 and the dopamine-D<sub>2</sub> receptor antagonist eticlopride decreased the locomotor activity in the open-field. The difference may be due to that in the experimental conditions: the previous study was performed under no habituation to the novel environment, while the locomotor response in this study was determined after a 3-hour habituation period. The lack of the effects of 5-HT<sub>1A</sub>, 5-HT<sub>2</sub>, and 5-HT<sub>3</sub> receptor antagonists was similar to the previous study without habituation (Tanyeri et al., 2013).

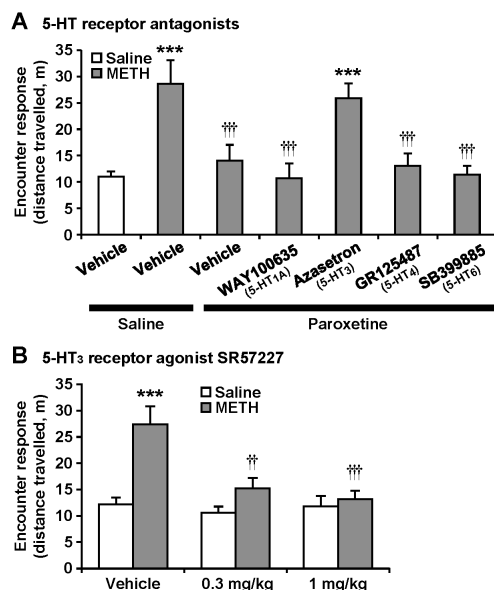
This study further pharmacologically characterized the encounter-induced hyperactivity in METH-sensitized mice. We showed that encounter-induced hyperactivity in METH-sensitized mice was attenuated by drugs with anxiolytic or antidepressant-like activity, such as diazepam, SSRIs, and the 5-HT<sub>1A</sub> receptor agonist osetozotan. Osetozotan, like buspirone, a nonbenzodiazepine anxiolytic, has an anxiolytic activity in preclinical models (Sakaue et al., 2001, 2003; Matsuda, 2013; Ago et al., 2014). These results are in agreement with previous observations in isolation-reared mice (Ago et al., 2013; Hasebe et al., 2015). Furthermore, the encounter-induced changes in prefrontal 5-HT and dopamine release and c-Fos expression are similar

between METH-sensitized and isolation-reared mice (Ago et al., 2013). However, the 5-HT receptor subtype involved in the effect of the SSRIs differed between METH-sensitized and isolation-reared mice. The effect of paroxetine was blocked by a 5-HT<sub>3</sub> receptor antagonist in METH-sensitized mice, while the effect of fluvoxamine was blocked by a 5-HT<sub>4</sub> receptor antagonist in isolation-reared mice (Hasebe et al., 2015). Therefore, it is likely that SSRIs attenuate the hyperactivity via activation of the 5-HT<sub>3</sub> receptor in METH-sensitized mice, in contrast to previous finding in isolation-reared mice that showed that the effect of SSRIs is mediated by the 5-HT<sub>4</sub> receptor. This observation may reflect a difference in the neurochemical basis between the 2 models. It is possible that isolation-reared mice have higher sensitivity of the 5-HT<sub>4</sub> receptor, while METH-sensitized mice have higher sensitivity of the 5-HT<sub>3</sub> receptor, but the exact mechanism is not known. The 5-HT<sub>4</sub> receptor has received considerable interest as a target for the treatment of depression (Lucas et al., 2007; Bockaert et al., 2008; Mendez-David et al., 2014). Conversely, Kondo et al. (2015) reported that the 5-HT<sub>3</sub> receptor plays a key role in exercise-induced antidepressant effects. The important role of the 5-HT<sub>3</sub> receptor is also demonstrated in the antiepileptic effect of SSRIs (Payandemehr et al., 2012; Alhaj et al., 2015).

Withdrawal from psychostimulant use is accompanied by symptoms of anxiety and depression, and the increased anxiety states are associated with greater conditioned place preference for cocaine in rats (Pelloux et al., 2009), and negative affect during withdrawal is thought to be a critical factor leading to craving and relapse in humans (Koob et al., 2004). Here, we demonstrated that the psychological stress encounter elicits



**Figure 7.** Effects of anxiolytic and antidepressant drugs on encounter-induced hyperactivity in methamphetamine (METH)-sensitized mice. The resident chronic METH- or saline-treated mice were i.p. injected with diazepam (1 mg/kg), osemozotan (0.3 mg/kg) (A), paroxetine (10 mg/kg), fluvoxamine (30 mg/kg) (B), or vehicle 30 minutes before the encounter with the intruder. Total distance travelled in the area near the partition during the 20-minute encounters was analyzed as an index of the encounter response. Results are expressed as the mean  $\pm$  SEM of 12 mice/group. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with vehicle/saline-injected saline-pretreated mice. † $P < .05$ , †† $P < .01$ , compared with vehicle/saline-injected METH-pretreated mice.



**Figure 8.** Involvement of 5-HT<sub>3</sub> receptor activation in the effect of paroxetine on encounter-induced hyperactivity. The resident chronic methamphetamine (METH)- or saline-treated mice were i.p. injected with paroxetine (10 mg/kg) (A), SR57227 (0.3, 1 mg/kg) (B), or saline 30 minutes before the encounter with the intruder. WAY100635 (1 mg/kg), azasetron (3 mg/kg), GR125487 (3 mg/kg), SB399885 (3 mg/kg), or vehicle was i.p. injected 30 minutes before the paroxetine treatment (A). Total distance travelled in the area near the partition during the 20-minute encounters was analyzed as an index of the encounter response. Results are expressed as the mean  $\pm$  SEM of 12 mice/group. \*\*\* $P < .001$ , compared with vehicle-injected saline-pretreated mice. † $P < .05$ , †† $P < .01$ , ††† $P < .001$ , compared with vehicle/saline-injected METH-pretreated mice.

hyperactivity during withdrawal in METH-sensitized mice, and the hyperactivity is attenuated by anxiolytics and antidepressants. The pharmacological results suggest that the behavior is associated with a state of anxiety or depression. It is likely that the psychological stress-induced hyperactivity is a factor leading to craving and relapse. Taken together, the neurochemical studies on the hyperactivity may contribute to development of pharmacotherapy for withdrawal symptoms of METH dependence.

In conclusion, the present study demonstrates that an encounter with an intruder, a psychological stress, elicits hyperactivity during withdrawal in METH-sensitized mice. Furthermore, we found that the hyperactivity is accompanied by an activation of prefrontal serotonergic and dopaminergic systems and is attenuated by anxiolytic and antidepressant drugs. The results of the present study imply that encounter-induced behavioral alteration is useful as a novel approach to investigate pharmacotherapy for withdrawal syndromes in METH-sensitized mice.

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## Statement of Interest

None.

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