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Role of Social Encounter-Induced Activation of Prefrontal Serotonergic Systems in the Abnormal Behaviors of Isolation-Reared Mice

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Isolation-reared male rodents show abnormal behaviors such as hyperlocomotion, aggressive behaviors, deficits of prepulse inhibition, and depression- and anxiety-like behaviors, but the neurochemical mechanism for the effects of psychological stress in these animals is not fully understood. This study examined the effects of social interactions between isolation-reared mice and intruder mice on brain monoaminergic systems. A cage was divided into two compartments by a mesh partition to prevent direct physical interactions. The 20-min encounter with an intruder elicited a restless and hyperexcitable state (hyperactivity) in male, but not in female, isolation-reared mice, whereas encounters with a sleeping intruder or a novel object did not. Although the encounter did not affect prefrontal neuronal-activity-marker c-Fos expression, dopamine (DA) levels, or serotonin (5-HT) levels in male group-reared mice or female isolation-reared mice, it increased prefrontal c-Fos expression, DA levels, and 5-HT levels in male isolation-reared mice. Furthermore, encounter-induced increases in c-Fos expression in the dorsal raphe nucleus and ventral tegmental area, but not in the nucleus accumbens shell, were much greater in isolation-reared than group-reared male mice. A 5-HT$_{1A}$ receptor agonist, a metabotropic glutamate 2/3 receptor agonist, and a gamma-aminobutyric acid A receptor agonist attenuated isolation-induced aggressive behaviors and encounter-induced hyperactivity, c-Fos expression in the prefrontal cortex and dorsal raphe nucleus, and increases in prefrontal 5-HT levels. These findings suggest that the prefrontal DA and 5-HT systems are activated by encounter stimulation in male isolation-reared mice, and the encounter-induced activation of 5-HT system triggers the induction of some abnormal behaviors in male isolation-reared mice. Furthermore, this study implies that the encounter stimulation-induced signal has a pharmacological significance.
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INTRODUCTION

Being raised in social isolation from early in life causes abnormal behaviors in adulthood, such as hyperlocomotion, aggressive behavior, deficits of prepulse inhibition, cognitive impairments, decreased social contact, and depression- and anxiety-like behavior in rodents (Ago *et al*, 2007, 2008; Fone and Porkess, 2008; Lukkes *et al*, 2009a, b; Zhao *et al*, 2009). Post-weaning social isolation is a useful model to study the effects of adverse early-life experiences on behavior and the neural mechanisms associated with isolation-induced changes in mood and anxiety (Lapiz *et al*, 2003; Lukkes *et al*, 2009c). Previous pharmacological studies have shown that serotonin 1A (5-HT$_{1A}$) receptor agonists and metabotropic glutamate 2/3 (mGlu$_{2/3}$) receptor agonists reverse isolation-rearing-induced abnormal behaviors (Ago *et al*, 2012; Jones *et al*, 2011; Maisonnette *et al*, 1993; Sakaue *et al*, 2001, 2003; Sánchez *et al*, 1993), but the exact neurochemical mechanisms are not known. There have been several studies on the neurochemical effects of isolation rearing, particularly focusing on the monoaminergic systems. These studies suggest that isolation rearing affects dopamine (DA) and 5-HT turnover in the brain regions, including the prefrontal cortex and nucleus accumbens (Eells *et al*, 2006; Heidbreder *et al*, 2000). Furthermore, some physical stressors or aversive stimuli cause a variety of changes in brain monoaminergic systems in isolation-reared rodents (Ago *et al*, 2002; Cabib *et al*, 2002; Fulford and Marsden, 1998a, 1998b, 2007; Hall *et al*, 1998; Jones *et al*, 1992; for reviews,

see Hall, 1998; Hall and Perona, 2012). However, the effects of social stress on brain monoaminergic systems in isolation-reared animals remain to be investigated.

As aggressive behaviors and social interaction deficits in isolation-reared rodents are induced by exposure to an intruder, it is likely that encounter with an intruder may produce neurobiological changes responsible for abnormal behaviors. There have been several neurochemical studies on resident–intruder interactions. In resident–intruder tests, van Erp and Miczek (2000) reported that encounters with the intruder increased prefrontal and accumbal DA and decreased cortical 5-HT levels in the resident aggressive rats. Anstrom et al (2009), using fast-scan cyclic voltammetry and multiunit recording techniques, reported that an aggressive encounter increases phasic DA transmission in the mesolimbic pathway in defeated rats. These neurochemical changes were observed both during and after the encounters, and it is likely that the changes are related to both psychological and physical stress. However, the neurochemical changes responsible for abnormal behaviors in aggressive rodents are not known.

In this study, we aimed to identify the primary neurochemical changes for induction of abnormal behaviors in isolation-reared mice. To address this issue, we used a cage that was divided into two compartments (large and small) by a mesh partition, and we examined the effects of intruder encounters on c-Fos expression and the levels of DA and 5-HT in the brains of resident mice reared in a group or in isolation. We used c-Fos expression to measure regional changes in brain activity (Koda et al, 2010; Kovács, 2008). As a control, we further examined female isolation-reared mice, because they do not develop hyper-aggression, unlike male isolation-reared mice (Pinna et al, 2005, 2008).

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. The ddY outbred male or female mice (SHIMIZU Laboratory Supplies, Kyoto, Japan) were weaned at 3 weeks of age and divided equally and concurrently into isolation- and group-housed conditions. The isolated male and female mice were individually housed for 6 weeks in wire-topped opaque polypropylene cages ($24 \times 17 \times 12 \, cm^3$), whereas control male mice were housed in groups (5–6 per cage) in the same-sized wire-topped clear plastic cages (Ago et al, 2007, 2008; Koda et al, 2008). All mice were housed under a standard 12-h light/dark cycle (lights on at 0800 hours) at a constant temperature of $22 \pm 1 \, ^\circ C$, with free access to food and water throughout the experiments. All experiments were conducted on 9-week-old male or female mice. The isolated male ddY-strain mice showed abnormal behaviors (Ago et al, 2007, 2008, 2012; Sakaue et al, 2001, 2003) consistent with those in C57BL/6J or ICR strain mice (Ibi et al, 2008; Koike et al, 2009; Varty et al, 2006; Zhang et al,

2012). We used 100 mice for behavioral analyses (Figures 1 ($n = 30$), 4a ($n = 20$) and 5 ($n = 50$)), 92 mice for c-Fos immunochemistry (Figures 2 ($n = 32$) and 6 ($n = 60$)), and 180 mice for microdialysis experiments (Figures 3 ($n = 60$), 4b ($n = 20$), 7 ($n = 50$) and 8 ($n = 50$)) in single use for each purpose. The drugs osemozotan (Mitsubishi Pharma, Yokohama, Japan), diazepam (Sigma, St Louis, MO, USA), LY379268 (Tocris Cookson, Bristol, UK), and pentobarbital (Nacalai Tesque, Kyoto, Japan) were used in this study. Osemozotan and diazepam were suspended in 0.5% w/v carboxymethylcellulose. LY379268 was dissolved in saline (0.9% solution of NaCl). Pentobarbital was dissolved in sterile water containing 10% ethanol. The drugs were injected intraperitoneally in a volume of 10 ml/kg. Mice were treated with a single injection of diazepam (1 mg/kg), osemozotan (0.3 mg/kg), or vehicle 30 min before the encounter with an unfamiliar male intruder (Figures 5–8). LY379268 (1 mg/kg) or saline was also given as a single-dose injection 60 min before the encounter (Figures 5–8). These doses of the drugs used here were determined by referring to previous studies: the doses potently inhibited isolation rearing-induced aggressive behavior, hyperactivity, deficits of prepulse inhibition, or cognitive impairments (Jones et al, 2011; Sakaue et al, 2001, 2003).

Social Encounter Stimulation and Behavioral Analysis

A 9-week-old male group-reared, male isolation-reared, or female isolation-reared mouse was placed in the large compartment of a novel clear Plexiglas cage ($30 \times 30 \times 35 \, cm^3$) divided by a mesh partition from a smaller compartment, under an ambient lighting of 100 lux (Figure 1a left). This allowed the animal to see, hear, and smell, but not to contact the neighbor physically. After a 3-h habituation period, an unfamiliar 9-week-old ddY mouse was introduced into the unoccupied small compartment as an intruder (male resident *vs* male intruder, and female resident *vs* female intruder). The resident and intruder mice were allowed to interact through the partition for 20 min, and then the intruder mouse was removed. To characterize the encounter stimulation, we also examined the effects of the introduction of a male intruder mouse under pentobarbital (40 mg/kg) anesthesia or a novel object (golf ball) for 20 min. The behaviors of the resident mouse were videotaped, and its locomotor path was automatically analyzed off-line using the ANY-maze video tracking software (Stoelting Company, Wood Dale, IL). The total time spent and locomotor activity (movement time) near the partition of the resident mouse (Figure 1a right; gray zone) was used to estimate the behavioral reactivity of mice to the intruder. The interaction times between the resident and intruder mice (time spent smelling, putting one or two paws on the partition, and putting noses into the holes toward the intruder) were also analyzed.

c-Fos Immunohistochemistry

The neuronal-activity-marker c-Fos was determined 2 h after the encounter in the brains of male group- and isolation-reared mice, as described previously (Ago et al, 2011; Koda et al, 2010). Mice that were not exposed to an intruder were used as controls (Figure 2). Each group- or
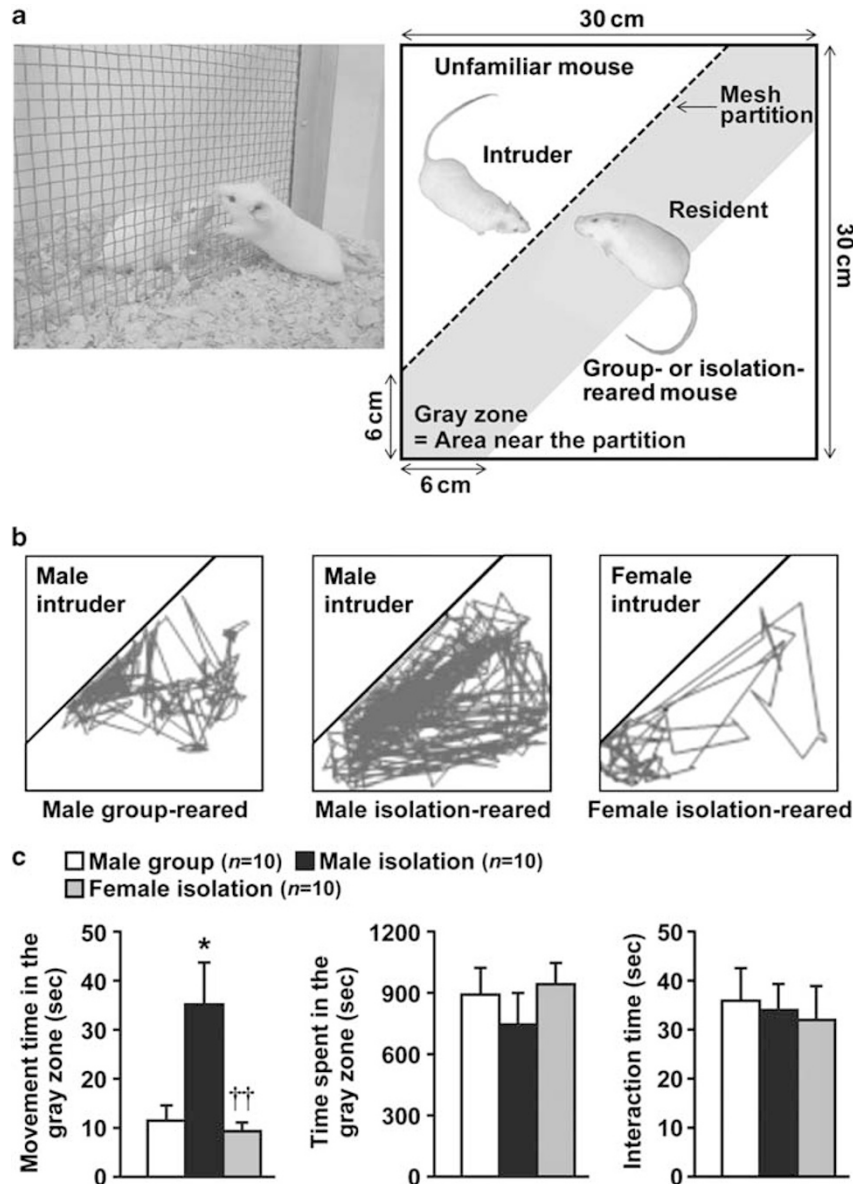
Figure 1 Effects of encounter stimulation on the behavior of mice. (a) Experimental design for the social encounter paradigm. A group- or isolation-reared mouse was individually placed into the large compartment of a novel clear Plexiglas cage divided by a mesh partition. An unfamiliar mouse was introduced into the unoccupied small compartment for 20 min. The gray zone was defined as the area near the partition. (b) Representative locomotor paths from resident male group-reared and isolation-reared mice and female isolation-reared mice during the 20-min encounters with the same sex intruders. (c) The locomotor activity and total time spent in the gray zone by the resident mice and the total time the resident and intruder mice spent interacting were analyzed according to the description in the Materials and Methods. The data are expressed as the mean ± SEM of 10 mice/group. *$p < 0.05$, compared with the male group-reared mice. ††$p < 0.01$, compared with the male isolation-reared mice.

isolation-reared mouse was deeply anesthetized with pentobarbital and perfused transcardially with saline, followed by a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS). The brain was fixed with 4% paraformaldehyde in PBS over 1 day and then transferred to 20% sucrose in PBS for 2 days. Serial 20-μm-thick coronal sections containing the prefrontal cortex ($+2.0$ through $+1.8$ mm with respect to bregma), nucleus accumbens ($+1.1$ through $+0.9$ mm with respect to bregma), amygdala (-1.3 through -1.5 mm with respect to bregma), ventral tegmental area (-2.8 through -3.0 mm with respect to bregma), and dorsal raphe nucleus (-4.4 through -4.6 mm with respect to bregma)

were cut using a cryostat microtome at $-20\,^{\circ}$C. The free-floating sections were preincubated for 30 min in 0.3% hydrogen peroxide in PBS to remove endogenous peroxidase activity. The sections were washed in PBS and then incubated with anti-c-Fos rabbit polyclonal primary antibodies (1:1000 dilution; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at room temperature. Subsequently, the primary incubation sections were washed in PBS and incubated in a secondary antibody solution containing biotinylated anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The sections were then incubated with avidin–biotin–horseradish peroxidase complex (Vectastain
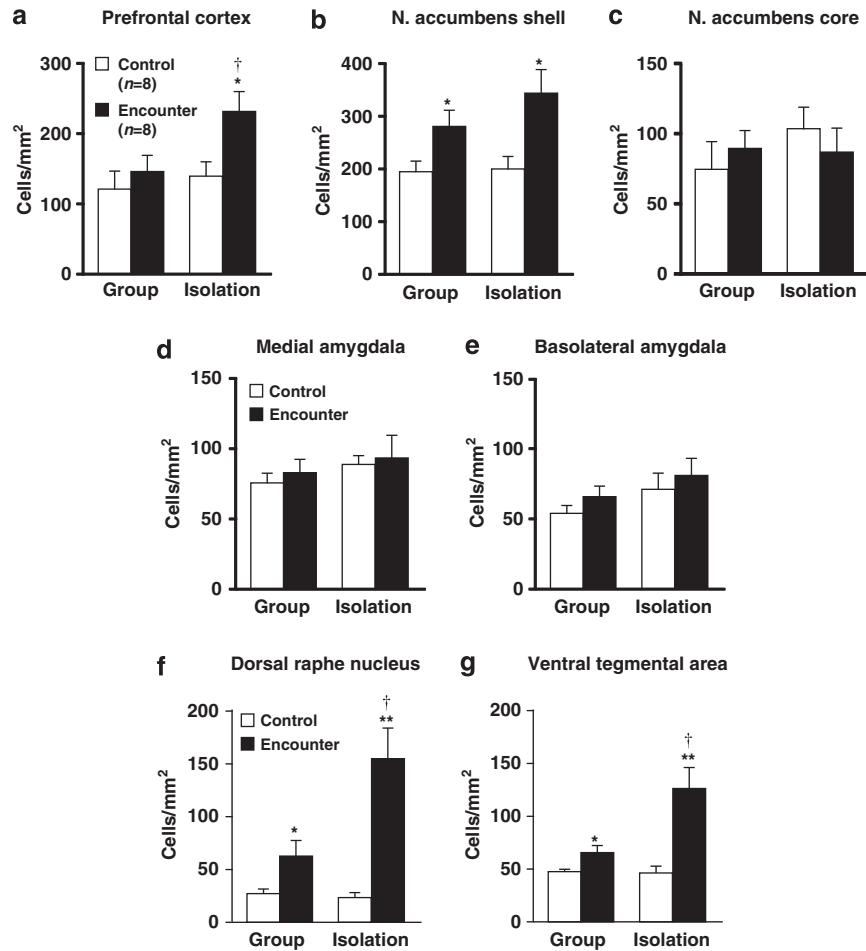
Figure 2 Effects of encounter stimulation on c-Fos expression in the prefrontal cortex (a), nucleus accumbens shell (b) and core (c), the medial (d) and basolateral (e) amygdala, dorsal raphe nucleus (f), and ventral tegmental area (g) of mice. Immunohistochemical localization of the neuronal-activity-marker c-Fos in the male group- and isolation-reared mice was determined 2 h after the encounter. Mice that were not exposed to an intruder were used as controls. The data are expressed as the mean ± SEM of 8 mice/group. *$p < 0.05$, **$p < 0.01$, compared with the control. †$p < 0.05$, compared with the encounter stress-challenged group-reared mice.

ABC kit; Vector Laboratories) for 30 min at room temperature. Brown cytosolic products were obtained by reaction with 3,3′-diaminobenzidine (Histofine SAB-PO (M) kit, Nichirei Bioscience, Tokyo, Japan). Sections were then mounted, dehydrated, and cover slipped. Four independent sections per animal containing the prefrontal cortex, nucleus accumbens, amygdala, dorsal raphe nucleus, or ventral tegmental area were selected. Then, c-Fos-positive nuclei were counted manually by experienced observers blinded to the rearing and encounter conditions of the animals, under bright-field illumination using a Keyence microscope (BIOREVO, BZ-9000) (Ago et al, 2011; Koda et al, 2010). The number of c-Fos-positive nuclei in each section was determined in a $500 \times 500 \, \mu m^2$ area in the left and right hemispheres and averaged using an ImageJ 1.41 software package (NIH, Bethesda, MD, USA). The mean of this average across four sections was then calculated for each subject.

In Vivo Microdialysis

Each mouse was anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and stereotaxically implanted with a guide cannula (one site per animal) for a dialysis probe (Eicom Corp., Kyoto, Japan) targeting the prefrontal cortex ($A \, +1.9 \, mm$, $L \, -0.5 \, mm$, $V \, -0.8 \, mm$, from the bregma and skull) or nucleus accumbens shell ($A \, +1.2 \, mm$, $L \, -0.5 \, mm$, $V \, -4.0 \, mm$) (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 a single injection of buprenorphine (0.1 mg/kg, i.p.; Ago et al, 2008, 2011). The active probe membranes were 3- and 1-mm long in the prefrontal cortex and nucleus accumbens, respectively. Two days after the surgery, the probe was perfused with Ringer's solution (147.2 mM NaCl, 4.0 mM KCl, and 2.2 mM CaCl₂, pH 6.0; Fuso Pharmaceutical Industries, Osaka, Japan) at a constant flow rate of 2 μl/min. A stabilization period of 3 h, which was identical to the 3-h habituation period in the clear Plexiglas cage described above, was established before the onset of the experiment. Microdialysis samples (20 μl) were collected every 10 min and injected immediately onto an HPLC column for simultaneous assay of DA and 5-HT, as previously reported (Ago et al, 2006, 2008). After the experiments, Evans Blue dye was microinjected through the cannula to histologically verify the position of the probe.

Only data from animals with correct probe placements were used in the analysis.

Statistics

All results are presented as the mean ± SEM. For the microdialysis experiments, the data were calculated as the percentage of change from basal dialysate concentrations, with 100% defined as the average of three samples before drug administration. These data were analyzed using two-way ANOVA for rearing/sex or treatment as the inter-subject factor and repeated measures with time as the intra-subject factor. For the c-Fos expression experiments, the data were analyzed using two-way ANOVA, followed by the Tukey–Kramer post-hoc test. For the behavioral experiments, the data were analyzed using Student's t-test or one-way ANOVA, followed by the Tukey–Kramer post hoc test. Statistical analyses were performed using the software package, Statview 5.0J, for Apple Macintosh (SAS Institute, Cary, NC). A P-value of < 0.05 was considered statistically significant.

RESULTS

Effects of Encounter Stimulation on the Behavior of Mice

We first examined the effects of encounter stimulation on the behavior of mice. A group-reared male, isolation-reared male, or isolation-reared female mouse was placed in a large compartment of a novel clear Plexiglas cage divided by a mesh partition, allowing the animal to see, hear, and smell but not to contact the neighbor physically (Figure 1a). After a habituation period, an unfamiliar intruder mouse was introduced into the unoccupied small compartment. Figure 1b shows the representative locomotor paths of the resident mice during the 20-min encounters. These locomotor patterns indicate that male isolation-reared mice were restless and hyperexcitable during the encounters, compared with male group-reared mice and female isolation-reared mice. Locomotor activities (movement time) of male isolation-reared mice in the area near the partition (Figure 1a, gray zone) were significantly higher than those of male group-reared mice and female isolation-reared mice ($F_{(2,27)} = 7.2$, $p < 0.01$; Figure 1c). Conversely, the total time spent in the gray zone by the resident mice ($F_{(2,27)} = 0.6$, $p > 0.05$) and the total time the resident and intruder mice spent interacting ($F_{(2,27)} = 0.1$, $p > 0.05$) did not differ between male group- and isolation-reared mice and female isolation-reared mice (Figure 1c).

Effects of Encounter Stimulation on Neuronal Activity

The neuronal-activity-marker c-Fos was determined 2 h after the social encounters in the prefrontal cortex, nucleus accumbens, amygdala, dorsal raphe nucleus, and ventral tegmental area of male group- and isolation-reared mice (Figure 2). Encounter stimulation caused a significant increase in the number of c-Fos-positive nuclei in the prefrontal cortex of male isolation-reared but not of male group-reared mice ($F_{(1,28)} = 5.4$, $p < 0.05$; Figure 2a). Encounter stress increased c-Fos expression in the nucleus

accumbens shell ($F_{(1,28)} = 12.4$, $p < 0.01$; Figure 2b), dorsal raphe nucleus ($F_{(1,28)} = 25.5$, $p < 0.0001$; Figure 2f), and ventral tegmental area ($F_{(1,28)} = 19.9$, $p < 0.001$; Figure 2g) of both male group- and isolation-reared mice. The increase in c-Fos expression in the dorsal raphe nucleus ($F_{(1,28)} = 8.4$, $p < 0.01$) and the ventral tegmental area ($F_{(1,28)} = 7.8$, $p < 0.01$), but not in the nucleus accumbens shell ($F_{(1,28)} = 0.8$, $p > 0.05$), of male isolation-reared mice was significantly higher than that in male group-reared mice. That is, the responses of the prefrontal cortex, dorsal raphe nucleus, and ventral tegmental area to encounter stimulation were higher in isolation-reared than in group-reared mice. Encounter stimulation did not affect c-Fos expression in the nucleus accumbens core or the central or basolateral nucleus of the amygdala (Figure 2c–e).

Effects of Encounter Stimulation on Extracellular DA and 5-HT Levels in the Prefrontal Cortex and Nucleus Accumbens Shell of Mice

We next examined the effects of social encounters on extracellular DA and 5-HT levels in the prefrontal cortex (Figure 3a) and nucleus accumbens shell (Figure 3b). The basal extracellular levels of DA and 5-HT in the prefrontal cortex and nucleus accumbens shell did not differ significantly between male group- and isolation-reared mice and female isolation-reared mice: the DA levels in the prefrontal cortex (the mean ± SEM, $n = 10$) were
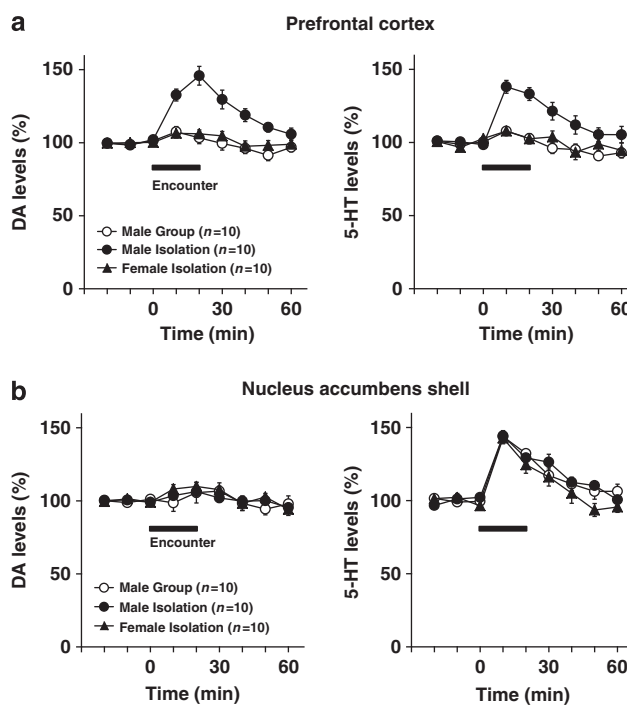
Figure 3 Effects of encounter stimulation on extracellular levels of DA and 5-HT in the prefrontal cortex (a) and nucleus accumbens shell (b) of mice. A male group- or isolation-reared mouse or female isolation-reared mouse was placed in a novel clear Plexiglas cage divided into two compartments by a mesh partition. After a 3-h habituation period, an unfamiliar intruder of the same sex as the resident was placed into the unoccupied compartment and the mice were allowed to interact for 20 min, as indicated by the horizontal bar. The data are expressed as the mean ± SEM of 10 mice/group.

0.38 ± 0.07 (male group), 0.39 ± 0.05 (male isolation), and 0.43 ± 0.03 pg/20 µl (female isolation); the 5-HT levels in the prefrontal cortex (the mean $\pm$ SEM, $n = 10$) were 0.78 ± 0.04 (male group), 0.72 ± 0.08 (male isolation), and 0.76 ± 0.13 pg/20 µl (female isolation); the DA levels in the nucleus accumbens shell (the mean $\pm$ SEM, $n = 10$) were 0.40 ± 0.04 (male group), 0.39 ± 0.07 (male isolation), and 0.39 ± 0.07 pg/20 µl (female isolation); the 5-HT levels in the nucleus accumbens shell (the mean $\pm$ SEM, $n = 10$) were 0.32 ± 0.08 (male group), 0.30 ± 0.05 (male isolation), and 0.36 ± 0.03 pg/20 µl (female isolation).

Encounter stress increased extracellular DA and 5-HT levels in the prefrontal cortex of male isolation-reared, but not of male group-reared or female isolation-reared, mice (significant interaction between the time and rearing/sex ($F_{(10,135)} = 4.3$, $p < 0.001$ for DA; $F_{(10,135)} = 3.4$, $p < 0.001$ for 5-HT); Figure 3a). Conversely, encounter stress increased extracellular 5-HT, but not DA, levels in the nucleus accumbens shell of all mice to a similar degree, as there was no significant difference in encounter-stress-induced 5-HT increases between the three groups ($F_{(10,135)} = 1.0$, $p > 0.05$; Figure 3b).

To characterize the encounter stress, we compared the effects of an encounter with a male intruder mouse with the introduction of a male intruder mouse under pentobarbital (40 mg/kg) anesthesia or a novel object (golf ball) for 20 min on the behaviors (Figure 4a) and prefrontal monoamine levels (Figure 4b) of the resident male isolation-reared mouse. In contrast to encounter with a conscious intruder mouse, male isolation-reared mice exhibited less activity

and spent the most time in the area near the partition (gray zone) during either comparison condition. In addition, exposure to an anesthetized mouse or exposure to a novel object did not affect extracellular DA and 5-HT levels in the prefrontal cortex.

Effects of Diazepam, Osemozotan, and LY379268 on Encounter-Induced Behavioral Changes in Male Isolation-Reared Mice

Encounter stimulation induced hyperactivity in male isolation-reared mice, suggesting that this hyperactivity is a behavioral response to an emotional state, such as anxiety or fear. Previous studies have shown that gamma-aminobutyric acid A (GABA$_A$) receptor agonists, 5-HT$_{1A}$ receptor agonists, and mGlu$_{2/3}$ receptor agonists attenuate isolation-rearing-induced aggressive behaviors and/or anxiety-like behaviors (Ago et al, 2012; Da Silva et al, 1996; Maisonnette et al, 1993; Sakaue et al, 2001; Sánchez et al, 1993; Wongwitdecha and Marsden, 1996). Thus, we next examined the effects of the GABA$_A$ receptor agonist diazepam, the 5-HT$_{1A}$ receptor agonist osemozotan, and the mGlu$_{2/3}$ receptor agonist LY379268 on encounter-induced behavioral changes in male isolation-reared mice (Figure 5). Figure 5a and c show representative locomotor paths of drug-treated male isolation-reared mice during the 20-min encounters. Diazepam (1 mg/kg, i.p.), osemozotan (0.3 mg/kg, i.p.) ($F_{(2,27)} = 6.7$, $p < 0.01$), and LY379268 (1 mg/kg, i.p.) ($p < 0.05$ by Student's t-test) inhibited encounter-induced hyperactivity. None of these drugs affected the total time spent in the gray zone or
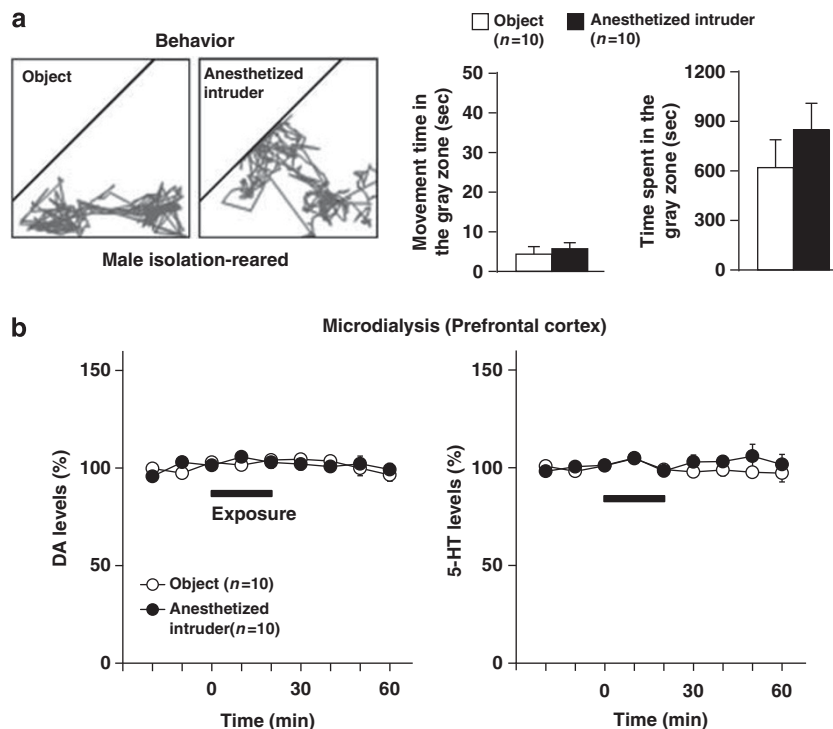

Figure 4 Effects of exposure to an anesthetized intruder or a novel object on behaviors (a) and extracellular levels of DA and 5-HT in the prefrontal cortex (b) of male isolation-reared mice. A male isolation-reared mouse was placed in a novel clear Plexiglas cage divided into two compartments by a mesh partition. After a 3-h habituation period, a pentobarbital-anesthetized (40 mg/kg, i.p.) male mouse or a novel object (golf ball) was placed into the unoccupied compartment for 20 min. (a) Representative locomotor paths of the resident mice during the 20-min exposure are shown in left panels. The locomotor activity of and total time spent in the gray zone by the resident mice during the exposure are shown in right panels. (b) The horizontal bars indicate the period of exposure to an anesthetized intruder or a novel object. The data are expressed as the mean $\pm$ SEM of 10 mice/group.
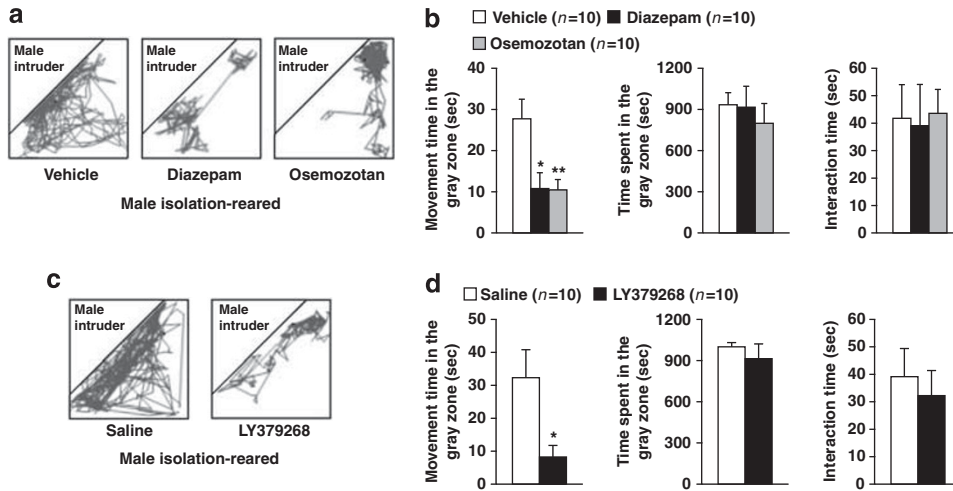
Figure 5 Effects of diazepam, osemozotan (upper panels), and LY379268 (lower panels) on encounter-induced behavioral changes in male isolation-reared mice. A male isolation-reared mouse was placed into the large compartment of a novel clear Plexiglas cage divided by a mesh partition. After a 3-h habituation period, the resident mice were intraperitoneally injected with vehicle, diazepam (1 mg/kg), or osemozotan (0.3 mg/kg) 30 min before the encounter with an unfamiliar male intruder for 20 min. Saline or LY379268 (1 mg/kg) was intraperitoneally injected 60 min before the encounter. (a, c) Representative locomotor paths of the resident mice during the 20-min encounters are shown. (b, d) The locomotor activity of and total time spent in the gray zone by the resident mice and the total time the resident and intruder mice spent interacting were analyzed according to the description in the Materials and Methods. The data are expressed as the mean ± SEM of 10 mice/group. *$p < 0.05$, **$p < 0.01$, compared with the vehicle or saline-treated mice.

the total time the resident and intruder mice spent interacting (Figure 5b and d).

Effects of Diazepam, Osemozotan, and LY379268 on Encounter-Induced c-Fos Expression in the Brain

We examined the effects of diazepam, osemozotan, and LY379268 on encounter-stress-induced c-Fos expression in the prefrontal cortex, nucleus accumbens shell, dorsal raphe nucleus, and ventral tegmental area of male group- and isolation-reared mice (Figure 6). The increases in prefrontal c-Fos expression during encounter stress were blocked by diazepam (1 mg/kg, i.p.), osemozotan (0.3 mg/kg, i.p.; significant interaction between the rearing and treatment: $F_{(2,30)} = 5.8$, $p < 0.01$), and LY379268 (1 mg/kg, i.p.; $F_{(1,20)} = 4.7$, $p < 0.05$) in male isolation-reared mice (Figure 6a). None of the drugs affected prefrontal c-Fos expression in male group-reared mice. Conversely, the increases in accumbal c-Fos expression during encounter stress were not blocked by diazepam, osemozotan, or LY379268 in either group (Figure 6b). All three drugs inhibited encounter-stress-induced increases in c-Fos expression in the dorsal raphe nucleus of male isolation-reared, but not of group-reared, mice ($F_{(2,30)} = 7.8$, $p < 0.01$ for diazepam and osemozotan; $F_{(1,20)} = 4.8$, $p < 0.05$ for LY379268; Figure 6c). Diazepam and LY379268, but not osemozotan, inhibited encounter-stress-induced increases in c-Fos expression in the ventral tegmental area of male isolation-reared mice (Figure 6d).

Effects of Diazepam, Osemozotan, and LY379268 on Encounter-Induced Increases in Prefrontal DA and 5-HT and Accumbal 5-HT Levels in Male Isolation-Reared Mice

To investigate further the possible involvement of encounter-induced neurochemical signal in induction of abnormal

behaviors in male isolation-reared mice, we examined the effects of diazepam, osemozotan, and LY379268 on encounter-induced increases in prefrontal DA and 5-HT levels (Figure 7) and accumbal 5-HT levels (Figure 8), respectively. In the prefrontal cortex, both diazepam (1 mg/kg, i.p.) and LY379268 (1 mg/kg, i.p.) blocked the increases in DA ($F_{(5,90)} = 2.4$, $p < 0.05$ for diazepam; $F_{(5,90)} = 3.8$, $p < 0.01$ for LY379268) and 5-HT ($F_{(5,90)} = 5.4$, $p < 0.001$ for diazepam; $F_{(5,90)} = 3.2$, $p < 0.01$ for LY379268) (from 0 to 60 min) levels seen in male isolation-reared mice during encounter stress. By contrast, osemozotan (0.3 mg/kg, i.p.) blocked encounter-stress-induced increases in 5-HT ($F_{(5,90)} = 8.6$, $p < 0.001$), but not DA ($F_{(5,90)} = 1.2$, $p > 0.05$), levels (from 0 to 60 min) in male isolation-reared mice. Basal extracellular levels of DA and 5-HT were not affected by either diazepam (from −30 to 0 min) or LY379268 (from −60 to 0 min), whereas basal extracellular levels of 5-HT ($F_{(2,36)} = 11.8$, $p < 0.001$), but not DA (from −30 to 0 min), were slightly decreased by osemozotan. In contrast to the prefrontal cortex, diazepam, osemozotan, and LY379268 did not affect encounter-stress-induced increases in extracellular 5-HT levels in the nucleus accumbens shell of male isolation-reared mice (from 0 to 60 min; Figure 8). Basal extracellular levels of 5-HT were not affected by either diazepam (from −30 to 0 min) or LY379268 (from −60 to 0 min), whereas those of 5-HT were slightly decreased by osemozotan in male isolation-reared mice ($F_{(2,36)} = 3.5$, $p < 0.05$; from −30 to 0 min). Basal extracellular levels of DA were not affected by any of the drugs tested.

DISCUSSION

The present study examined whether encounter stimulation produces neurochemical changes that induce abnormal behaviors in isolation-reared mice. Previous studies in resident–intruder tests have shown that DA and 5-HT
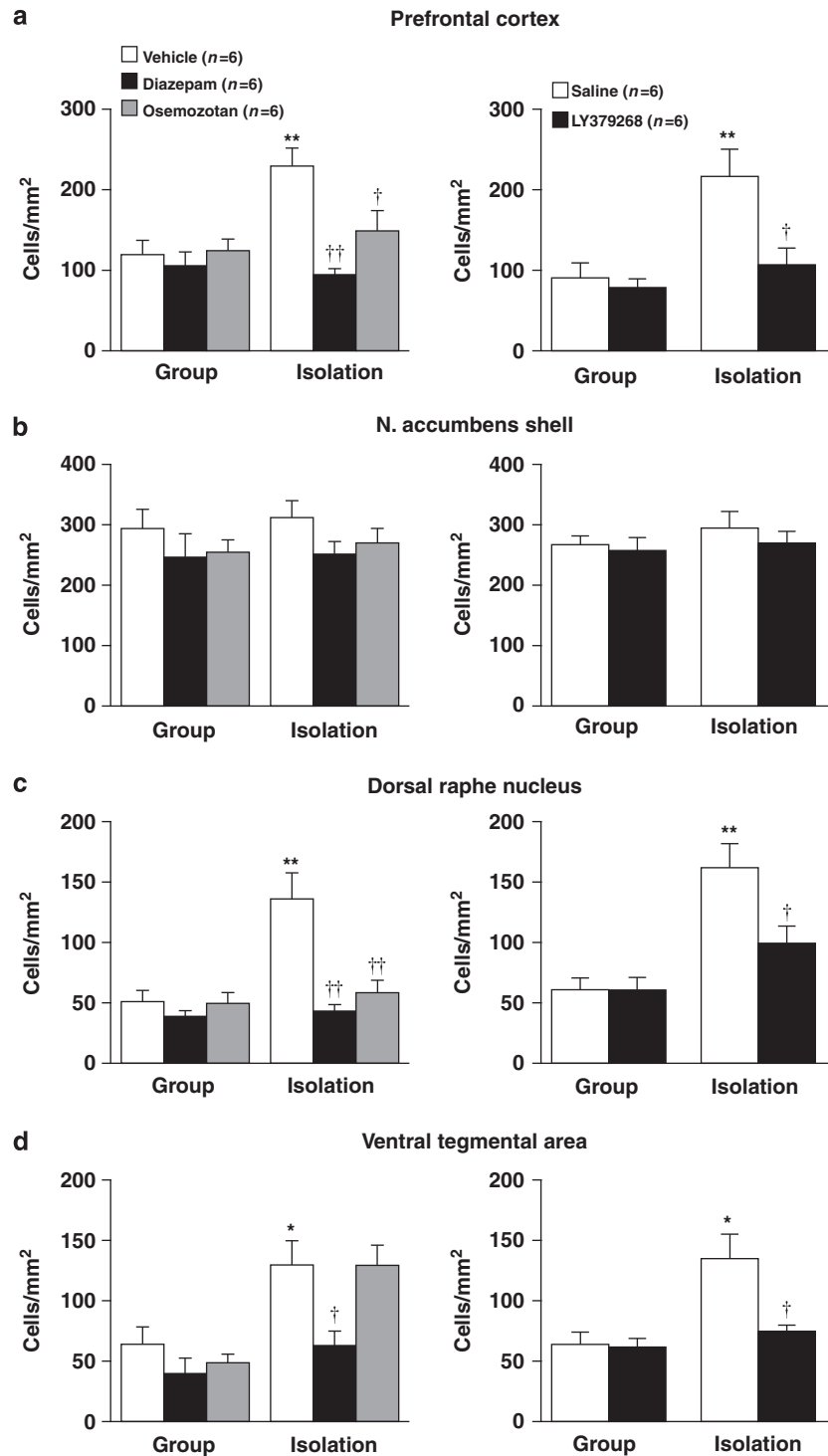
Figure 6 Effects of diazepam, osemozotan (left panels), and LY379268 (right panels) on encounter-induced c-Fos expression in the prefrontal cortex (a), nucleus accumbens shell (b), dorsal raphe nucleus (c), and ventral tegmental area (d) of male group- and isolation-reared mice. A mouse was placed into the large compartment of a novel clear Plexiglas cage divided by a mesh partition. After a 3-h habituation period, the resident mice were intraperitoneally injected with vehicle, diazepam (1 mg/kg), or osemozotan (0.3 mg/kg) 30 min before the encounter with an unfamiliar male intruder for 20 min. Saline or LY379268 (1 mg/kg) was intraperitoneally injected 60 min before the encounter. Immunohistochemical localization of the neuronal-activity-marker c-Fos was determined 2 h after the encounter. The data are expressed as the mean ± SEM of 6 mice/group. $*p < 0.05$, $**p < 0.01$, compared with the vehicle or saline-treated group-reared mice. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, compared with the vehicle or saline-treated isolation-reared mice.

systems are involved in aggressive and defeat behaviors during the encounter (Anstrom et al, 2009; Ferrari et al, 2003; Tidey and Miczek, 1996; van Erp and Miczek, 2000). It appears that the effects of the encounter in resident-intruder tests are related to both psychological and physical stress. In this study, we designed the encounter stimulation to occur through a mesh partition. This experimental procedure avoids aggressive contact (ie, biting
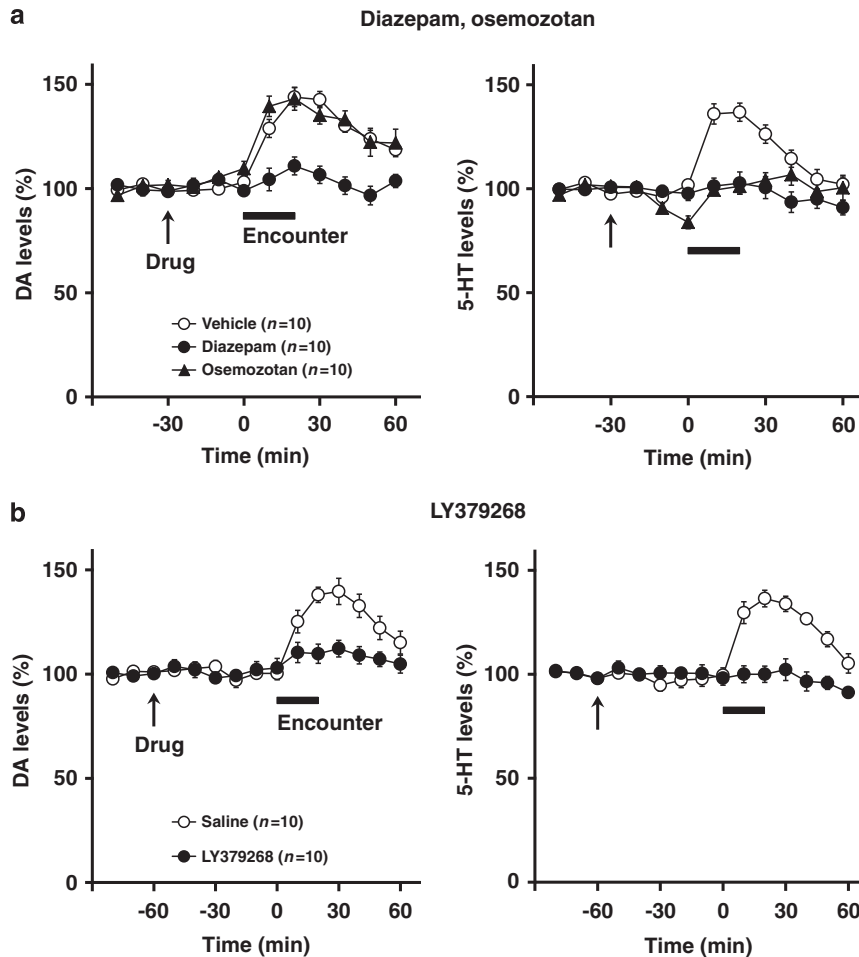
Figure 7 Effects of diazepam and osemozotan (a), and LY379268 (b) on encounter-induced increases in DA and 5-HT levels in the prefrontal cortex of male isolation-reared mice. A male isolation-reared mouse was placed into the large compartment of a novel clear Plexiglas cage divided by a mesh partition. After a 3-h habituation period, the resident mice were intraperitoneally injected with vehicle, diazepam (1 mg/kg), or osemozotan (0.3 mg/kg) 30 min before the encounter (a) and were intraperitoneally injected with saline or LY379268 (1 mg/kg) 60 min before the encounter (b) (arrow). After the drug treatment, an unfamiliar male intruder was placed into the unoccupied compartment and the mice were allowed to interact for 20 min, as indicated by the horizontal bar. The data are expressed as the mean ± SEM of 10 mice/group.

attacks, wrestling, lateral threats) from isolation-reared mice to an intruder mouse. However, the mice were able to see, hear, and smell their neighbor. Thus, a social encounter with an unfamiliar conspecific through a mesh partition is considered a form of psychological stress, and it is useful for studies of the neurochemical changes that may be involved in the abnormal behaviors of isolation-reared animals (Ago *et al*, 2012; Fone and Porkess, 2008; Lukkes *et al*, 2009a, b; Pinna *et al*, 2008).

In this study, the total time that the resident mice spent near the partition and the total time the resident and intruder mice spent interacting did not differ between the male group-reared, female isolation-reared, and male isolation-reared mice (Figure 1c). This suggests that the attention of the resident mice to the intruder is similar between these three groups. Under the conditions used, we found that encounter stimulation increased locomotor activity in male isolation-reared mice but not in male group-reared or female isolation-reared mice (Figure 1b and c). Furthermore, we demonstrated that the encounter with the sleeping intruder or exposure to a novel object did not increase locomotor activity in male isolation-reared

mice (Figure 4a). As social isolation does not induce aggressive behavior in female mice (Pinna *et al*, 2003, 2005, 2008), the present findings indicate that encounter-induced behavioral responses to emotional states, such as anxiety or fear, are observed selectively in male isolation-reared mice.

As the neural circuits that include the nucleus accumbens, amygdala, and prefrontal cortex are known to have crucial roles in emotions, motivation, and reward (Cardinal *et al*, 2002; Carlezon and Thomas, 2009; Ishikawa *et al*, 2008; Kalivas and Nakamura, 1999), we examined the effects of encounter stimulation on c-Fos expression in these brain regions. Encounter stimulation increased c-Fos expression in the nucleus accumbens shell of group- and isolation-reared mice to a similar degree. Activation of nucleus accumbens increases exploratory and social play behaviors in rats (Falowski *et al*, 2011; Trezza *et al*, 2011; van Kuyck *et al*, 2007). Thus, enhanced neuronal activity in the nucleus accumbens shell may contribute to social interactions in male group- and isolation-reared mice. It should be noted that encounter stimulation increased prefrontal c-Fos expression in male isolation-reared mice, whereas it did not affect the c-Fos expression in group-reared mice.
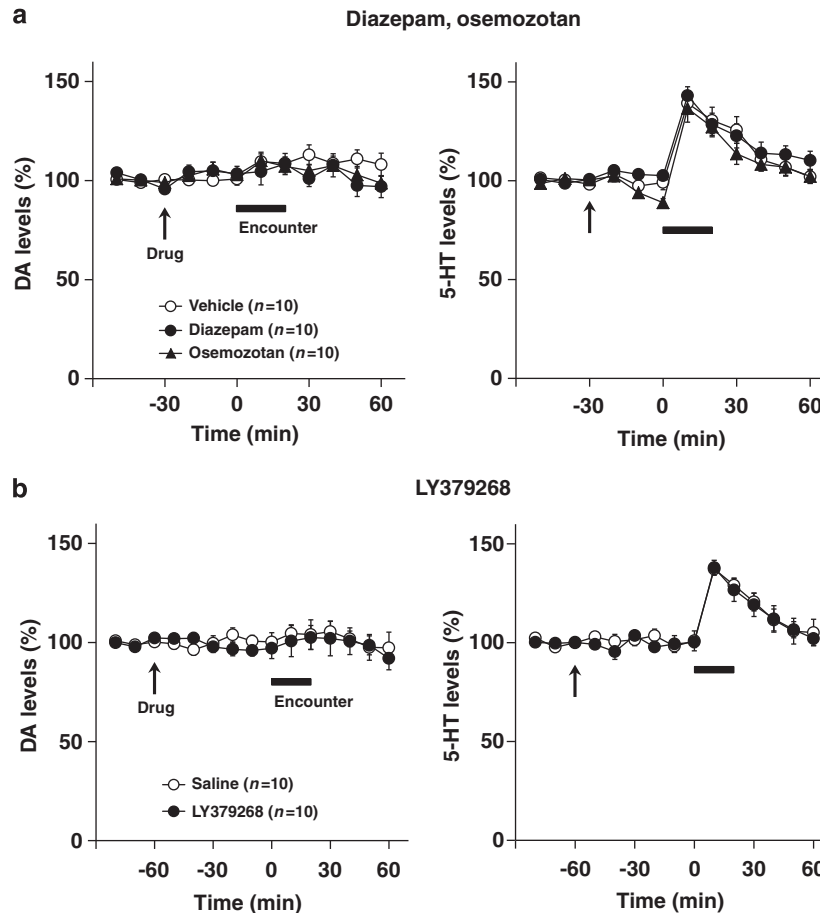
Figure 8 Effects of diazepam and osemozotan (a), and LY379268 (b) on encounter-induced increases in extracellular 5-HT levels in the nucleus accumbens shell of male isolation-reared mice. A male isolation-reared mouse was placed into the large compartment of a novel clear Plexiglas cage divided by a mesh partition. After a 3-h habituation period, the resident mice were intraperitoneally injected with vehicle, diazepam (1 mg/kg), or osemozotan (0.3 mg/kg) 30 min before the encounter (a) and were intraperitoneally injected with saline or LY379268 (1 mg/kg) 60 min before the encounter (b) (arrow). After the drug treatment, an unfamiliar male intruder was placed into the unoccupied compartment and the mice were allowed to interact for 20 min, as indicated by the horizontal bar. The data are expressed as the mean ± SEM of 10 mice/group.

Furthermore, isolation-reared male mice showed enhanced c-Fos expression in response to encounter stimulation in the ventral tegmental area and dorsal raphe nucleus. The prefrontal cortex is innervated by dopaminergic fibers from the ventral tegmental area and serotonergic fibers from dorsal raphe nucleus. Thus, it is likely that increased prefrontal neuronal activity via activation of the dorsal raphe nucleus and ventral tegmental area triggers abnormal behaviors in male isolation-reared mice.

In agreement with the results of c-Fos expression, encounter stimulation increased extracellular DA and 5-HT levels in the prefrontal cortex of male isolation-reared, but not male group-reared or female isolation-reared, mice. We found that an intruder anesthetized with pentobarbital or exposure to a novel object did not affect extracellular DA and 5-HT levels in the prefrontal cortex of male isolation-reared mice. This suggests that interactions between the resident mouse and an intruder mouse are necessary for these increases in prefrontal DA and 5-HT in male isolation-reared mice. Previous studies have shown that prefrontal DA levels are higher during or after an aggressive encounter in both attacking and defending rats (Anstrom et al, 2009; Tidey and Miczek, 1996; van Erp and

Miczek, 2000). Furthermore, exposure to tests of 'anxiety', such as the social interaction test, causes an increase in hippocampal 5-HT release in rats from the more anxious Wistar strain, but not in rats from the less anxious Sprague–Dawley strain (Rex et al, 2004). These studies suggest that neurochemical changes are involved in encounter-induced behaviors, although these neurochemical changes may be induced by both psychological and physical stress. Under these circumstances, we performed pharmacological experiments to examine whether encounter-induced increases in prefrontal DA and 5-HT release are indeed the neurochemical basis for induction of abnormal behaviors in male isolation-reared mice.

In these experiments, we used the GABA$_A$ receptor agonist diazepam, the 5-HT$_{1A}$ receptor agonist osemozotan, and the mGlu$_{2/3}$ receptor agonist LY379268. Diazepam is a potent anxiolytic (do-Rego et al, 2006) and attenuates aggressive behavior (Sakaue et al, 2001; Wongwitdecha and Marsden, 1996) and anxiety-like behavior in the elevated plus-maze (Da Silva et al, 1996) in isolation-reared rodents. Osemozotan and mGlu$_{2/3}$ receptor agonists also inhibit aggressive behavior and anxiety-like behavior in the elevated plus-maze, and they reverse the deficits of prepulse

inhibition in isolation-reared animals (Ago *et al*, 2012; Maisonnette *et al*, 1993; Sakaue *et al*, 2001, 2003; Sánchez *et al*, 1993). The present study demonstrated that encounter-induced hyperactivity and increased prefrontal 5-HT levels were blocked by diazepam, osemozotan, and LY379268. In contrast, encounter-induced increases in prefrontal DA levels were blocked by diazepam and LY379268, but not osemozotan. Consistent with these findings, encounter-induced increases in c-Fos expression in the prefrontal cortex and dorsal raphe nucleus were also blocked by all the three drugs in male isolation-reared mice. Concerning the mechanisms for the effects of the drugs, serotonergic neurons in the dorsal raphe nucleus are regulated not only by presynaptic 5-HT$_{1A}$ receptors but also by inhibitory GABA and excitatory glutamate neurons (Celada *et al*, 2001; Liu *et al*, 2000), and mGlu$_{2/3}$ receptor agonists may inhibit glutamate release (Cartmell and Schoepp, 2000). In the ventral tegmental area, dopaminergic neurons are regulated by the inhibitory and excitatory neurons (Geisler and Wise, 2008). Then, it is conceivable that diazepam and LY379268 inhibit prefrontal 5-HT and DA release and osemozotan inhibits prefrontal 5-HT release. Furthermore, we found that encounter stimulation increased extracellular 5-HT, but not DA, levels in the nucleus accumbens shell of all the three groups. However, none of the drugs affected encounter-induced 5-HT release or c-Fos expression in the nucleus accumbens shell of male isolation-reared mice. Taken together, it is likely that prefrontal 5-HT release is the signal that induces abnormal behaviors in response to encounter stimulation in male isolation-reared mice.

In conclusion, this study demonstrates that male isolation-reared mice show enhanced encounter-induced increases in c-Fos expression in the prefrontal cortex, dorsal raphe nucleus, and ventral tegmental area. Consistent with these observations, microdialysis showed that the encounter stimulation increased prefrontal DA and 5-HT levels in male isolation-reared mice. In addition, we found that the increased prefrontal 5-HT levels were blocked by diazepam, osemozotan, and LY379268, whereas increased prefrontal DA levels were only blocked by diazepam and LY379268. Taken together, it is likely that encounter stimulation specifically activates prefrontal DA and 5-HT systems, and that the 5-HT system has a key role in the encounter-induced behaviors in male isolation-reared mice. We also speculate that the activation of prefrontal 5-HT system triggers the induction of some abnormal behaviors in isolation-reared mice under conditions of psychological stress, such as anxiety-related tests and a startle test (prepulse inhibition). As isolation-reared rodents are considered to be a model of schizophrenia, anxiety, or mood disorders, the present findings imply that serotonergic projections from the dorsal raphe to the frontal cortex are a potential target for treatment of psychiatric disorders.

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DISCLOSURE

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