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The Antidepressant Effects of an mGlu2/3 Receptor Antagonist and Ketamine Require AMPA Receptor Stimulation in the mPFC and Subsequent Activation of the 5-HT Neurons in the DRN

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We have reported the antidepressant effects of both metabotropic glutamate 2/3 (mGlu2/3) receptor antagonists and ketamine in several animal models, and proposed that serotonergic (5-HTergic) transmission is involved in these actions. Given that the projections from the medial prefrontal cortex (mPFC) to the dorsal raphe nucleus (DRN), where the majority of serotonin (5-HT) neurons exist, are reportedly involved in the antidepressant effects, in this study, we investigated using the forced swimming test (FST) of C57BL/6J male mice, the role of 5-HT neurons in the DRN regulated by the mPFC–DRN projections in the antidepressant effects of an mGlu2/3 receptor antagonist, LY341495, and ketamine. Following systemic administration/microinjection into the mPFC, both LY341495 and ketamine were found to exert antidepressant effects in the FST, and the effects were attenuated by depletion of 5-HT by treatment with an inhibitor of 5-HT synthesis, PCPA. The antidepressant effects of LY341495 and ketamine were also blocked by systemic administration/microinjection into the mPFC of an AMPA receptor antagonist, NBQX. Moreover, systemic administration/microinjection into the mPFC of LY341495 and ketamine significantly increased the c-Fos expression in the 5-HT neurons in the DRN, and the effect of systemic administration of these drugs on the neuronal c-Fos expression was attenuated by microinjection of NBQX into the mPFC. Our findings suggest that activation of 5-HT neurons in the DRN regulated by stimulation of the AMPA receptor in the mPFC may be involved in the antidepressant effects of an mGlu2/3 receptor antagonist and ketamine.
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INTRODUCTION

Modulation of the glutamatergic system has emerged as an effective approach to treat depression that is represented by ground-breaking findings of a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, ketamine. A single injection of ketamine has been reported to yield the rapid and sustained antidepressant effects not only in patients with major depressive disorder but also in patients with treatment-resistant depression (Berman *et al*, 2000; Ibrahim *et al*, 2011; Zarate *et al*, 2006). However, ketamine also has undesirable side effects, such as psychotomimetic/dissociative symptoms and abuse potential (Krystal *et al*, 2013), that stimulated research activity to investigate the mechanisms underlying its antidepressant effects so that alternatives with fewer side effects may be identified. To date, increases in synaptic protein synthesis and thereby spine density have been proposed to mediate the antidepressant effects of ketamine (Li *et al*, 2010), presumably through stimulation of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor that leads to the subsequent activation of brain-derived neurotrophic factor (BDNF)/tropomyosin-related kinase B (TrkB)/mammalian target of rapamycin (mTOR) signaling and inhibition of eukaryotic elongation factor 2 kinase (Autry *et al*, 2011; Krystal *et al*, 2013; Li *et al*, 2010).

Among the possible alternatives to ketamine are metabotropic glutamate 2/3 (mGlu2/3) receptor antagonists. We have demonstrated the antidepressant effects of mGlu2/3 receptor antagonists in several animal models of depression (Chaki *et al*, 2004; Karasawa *et al*, 2005; Pałucha-Poniewiera *et al*, 2010), including a model that is depression refractory to current medications (Koike *et al*, 2013a). In addition, we and others have found evidence to suggest that mGlu2/3 receptor antagonists share some of the neural and synaptic mechanisms underlying their antidepressant effects with ketamine, such as AMPA receptor stimulation and subsequent activation of the TrkB receptor and mTOR signalings, that increase the levels of synaptic proteins in the prefrontal cortex (PFC) (Dwyer *et al*, 2012; Koike *et al*, 2011a; Koike *et al*, 2013b;

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Pałucha-Poniewiera *et al*, 2010). Therefore, like ketamine, mGlu2/3 receptor antagonists may have the potential to exert the rapid and sustained antidepressant effects and, indeed, an mGlu2/3 receptor antagonist has been shown to exert the rapid and sustained antidepressant effects in the chronic unpredictable stress model (Dwyer *et al*, 2013).

In addition to the involvement of synaptic plasticity, involvement of the serotonergic (5-HTergic) systems as well in the antidepressant effects of an mGlu2/3 receptor antagonist and ketamine has been suggested. We previously reported that mGlu2/3 receptor antagonists increased the firing of the serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) as well as enhanced the release of 5-HT in the medial PFC (mPFC) through activation of the AMPA receptor (Karasawa *et al*, 2005; Kawashima *et al*, 2005). Similarly, ketamine has been reported to increase the release of 5-HT in the mPFC of rats and 5-HT_{1B} receptor binding in the nucleus accumbens and ventral pallidum of rhesus monkeys, both actions mediated by activation of the AMPA receptor (Nishitani *et al*, 2014; Yamanaka *et al*, 2014). Moreover, we recently reported that 5-HTergic transmission was involved in the actions of both an mGlu2/3 receptor antagonist and ketamine in the novelty-suppressed feeding test (NSFT), presumably in an AMPA receptor-dependent manner (Fukumoto *et al*, 2014). However, the precise neural mechanisms mediating 5-HTergic transmission in the action of both drugs have not yet been investigated.

The 5-HT neurons in the DRN are regulated by neural projections from various brain regions including the PFC (Pollak Dorocic *et al*, 2014). Recently, it was reported that although selective stimulation, using an optogenetic technique, of the DRN neurons regulated by mPFC projections induced potent antidepressant effects in rats, activation of the entire population of DRN neurons did not induce the antidepressant effects (Warden *et al*, 2012). Thus, the 5-HTergic systems selectively modulated by the mPFC–DRN projections may be involved in the antidepressant effects. This hypothesis was underpinned by the finding that deep brain stimulation (DBS) of the mPFC exerted an antidepressant effect that was abolished by 5-HT depletion (Hamani *et al*, 2010). Collectively, these findings suggest that activation of a subset of 5-HT neurons in the DRN modulated by mPFC projections may have an important role in the antidepressant effects of mGlu2/3 receptor antagonists and ketamine.

Therefore, in this study, we first investigated whether mPFC might be involved in the antidepressant effects of an mGlu2/3 antagonist and ketamine using the forced swimming test (FST), and then investigated the role of the 5-HT neurons in the DRN selectively regulated by mPFC projections in the antidepressant effects of an mGlu2/3 receptor antagonist and ketamine.

MATERIALS AND METHODS

Animals and Housing

Eight- or nine-week-old male C57BL/6J mice (Charles River Laboratories, Yokohama, Japan) were used for all the experiments. The animals were maintained in controlled temperature (23 ± 3 °C) and humidity (50 ± 20%) conditions under a 12-h light/dark cycle (lights on at 0700 h). Food and water were provided *ad libitum*. All the studies were performed according to the guidelines of the Taisho Pharmaceutical animal care committee and met the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal Experiments (1987).

Drug Administration

For systemic administration, LY341495 (Tocris Cookson, Bristol, UK) (mGlu2/3 receptor antagonist) was dissolved in 1/15 M phosphate buffer (pH 8.0). Ketamine (Veterinary Ketalar 50; Sankyo Yell Pharmaceutical, Tokyo, Japan) (NMDA receptor antagonist) was diluted with saline. Paroxetine hydrochloride (Toronto Research Chemicals, Toronto, ON, Canada) (selective serotonin reuptake inhibitor) was dissolved in distilled water. NBQX (Tocris Cookson) (AMPA receptor antagonist) was suspended in saline. *para*-Chlorophenylalanine (PCPA; Sigma-Aldrich, St Louis, MO) (5-HT synthesis inhibitor) was suspended in 0.5% methylcellulose (0.5% MC). LY341495 solution and ketamine solution were diluted using Ringer's solution (147 mM NaCl, 4 mM KCl, and 1.2 mM $CaCl_2$) before being used for the intracerebral microinjection. NBQX was dissolved in DMSO, and diluted with Ringer's solution before being used for the intracerebral microinjection. LY341495 (0.1, 0.3, 1, and 3 mg/kg), ketamine (3, 10, and 30 mg/kg), and paroxetine (1, 3, and 10 mg/kg) were administered intraperitoneally (i.p.) 30 min or 24 h before the test. NBQX (1, 3, and 10 mg/kg) was administered subcutaneously (s.c.) 35 min before the test. PCPA (300 mg/kg) was administered i.p. twice daily (at 0700–1100 h and 1600–1900 h) for 3 consecutive days, and the tests were conducted 18 h after the final administration. All the drugs were injected at a volume of 10 ml/kg body weight for the systemic administration experiments. The doses for the systemic administration of LY341495, ketamine, NBQX, and PCPA were selected based on previous reports (Fukumoto *et al*, 2014; Koike *et al*, 2013b). The dose for the systemic administration of paroxetine was selected according to the observation in a previous study (Sugimoto *et al*, 2011). For the intracerebral microinjection experiments, LY341495 (0.003 and 0.03 pmol/0.1 μl/side) or ketamine (0.3 and 3 nmol/0.1 μl/side) was injected 30 min or 24 h before the test, and NBQX (0.01 and 0.03 nmol/0.1 μl/side) was injected 35 min before the test. The doses for the intracerebral microinjection of LY341495, ketamine, and NBQX were selected based on the observations in previous studies (Iijima *et al*, 2013; López-Gil *et al*, 2007; López-Gil *et al*, 2012; Nishitani *et al*, 2014).

Microinjection

Each mouse was anesthetized with pentobarbital (50 mg/kg, i.p.) and fixed to a brain stereotaxic apparatus (Narishige Instruments, Tokyo, Japan). For the injection into the mPFC, the brains were implanted with guide cannulas (Eicom, Kyoto, Japan) bilaterally, so that the tips were positioned near the mPFC (anteroposterior, 2.0 mm from bregma; lateral, ± 1.4 mm; ventral, -2.3 mm; angle, 20°). The cannulas were held in place with dental cement. A dummy cannula was inserted into the guide cannula to prevent clogging. Microinjection of LY341495, ketamine, and NBQX

was performed on day 2 or 3 after the surgery. Before the microinjections, the dummy cannulas were removed from the guide cannula, and a 28-gauge injection cannula, extending 0.5 mm from the tip of the guide cannula, was inserted. The injection cannula was connected via a Teflon tubing to a microsyringe (Hamilton, Reno, NV) driven by a micro infusion pump (Harvard Apparatus, Holliston, MA). Injections of LY341495, ketamine, and NBQX were performed over 2 min at the rate of 0.05 µl/min. The injection cannulas were left in position for an additional 2 min before being withdrawn. After the behavioral test, Evans blue was infused, followed by preparation of coronal sections to confirm the locations of the cannula tips. The locations of the cannula tips are shown in Figures 2e–h and 3g and h and Supplementary Figures S2c and d and S3.

Forced Swimming Test

The FST was performed by a previously reported method, with some modification (Ago *et al*, 2013). Although immobility in the FST is a controversial measure of depressive-like behavior, it is a useful screen for compounds that are effective for the treatment of depression. Thus, we used the FST in the present study and evaluated the effects of the compounds by measuring the immobility time of the animals in the test. For the test, the mice were placed in a swim tank for 6 min on day 1 to induce a state of helplessness, and then placed back in the swim tank for 6 min on day 2 to measure the immobility time. The swimming sessions were conducted by placing the mice in cylinders (24 cm height × 17 cm diameter) containing water (25 ± 1 °C) up to a height of 13 cm, so that the mice could not support themselves by touching the bottom of the tank with their paws. The FST was conducted between 0800 and 1700 h. The water in the cylinders was changed after every trial. Test sessions were videotaped from the front of the cylinders for later scoring by a scorer. The total duration of immobility during a 6-min test session was measured by an observer blinded to the treatment conditions. Immobility was defined as floating in the water without struggling and only making movements necessary to keep the head above the water.

Measurement of the Spontaneous Locomotor Activity

Mice were housed individually in transparent acrylic cages (φ30 cm × 30 cm). Each compound was injected at designated time point, and the spontaneous locomotor activities were recorded for 60 min afterward using a SCANET apparatus (Neuroscience, Tokyo, Japan) placed in a sound-proof box.

Tissue Processing

Mice were administered LY341495 (0.03 pmol/0.1 µl/side, 1 mg/kg, s.c.) or ketamine (3 nmol/0.1 µl/side, 30 mg/kg, s.c.) 90 min before the perfusion, and NBQX (0.03 nmol/0.1 µl/side) 95 min before the perfusion. The administration time of these drugs was selected based on the findings of a previous study (Nowak *et al*, 2012). Following induction of inhalation anesthesia (Isoflurane; Mylan Pharmaceutical, Tokyo, Japan), the mice were perfused with 0.1 M phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, post-fixed (overnight), cryoprotected in a 30% sucrose solution (in PBS), and stored at 4 °C until they sank. Coronal sections (2 mm) throughout the DRN (bregma − 4.00 to − 6.00) were embedded in OCT compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen.

Immunofluorescence Studies

Cryosections (40 µm) at 1 : 3 (120 µm) intervals through the DRN between − 4.48 and − 4.84 mm from the bregma (2 serial sections for a region) were prepared. The expression of c-Fos in the 5-HT cell bodies in the DRN was studied by double immunofluorescence. Sections were washed thrice with PBS for 10 min each and blocked with PBS/0.3% Triton X-100/5% donkey serum for 1 h at room temperature before incubation overnight with rabbit anti-c-Fos antibody (1 : 500, Abcam, Cambridge, MA) and goat anti-TPH2 antibody (1 : 500, Abcam) at 4 °C. After washing, the sections were incubated with the fluorescent 594 donkey anti-rabbit IgG secondary antibody and 488 donkey anti-goat IgG secondary antibody (1 : 200, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. The sections were then mounted on slides, and coverslipped.

Image Processing and Quantification

In the DRN, sections were assessed for the number of single 5-HT-positive cells and double-labeled cells for 5-HT and c-Fos using a confocal microscope at a magnification of × 40 (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany); the cells were counted using the ImageJ software (National Institutes of Health, Bethesda, MD) by an observer who was blinded to the experimental treatment allocations of each mouse. Cell counts for a region are based on cell counts from a single brain section from each mouse from each treatment group. There was no significant variation of total number of 5-HT-positive cells within each treatment group. Colocalization of c-Fos immunoreactivity with 5-HT in the cell bodies was confirmed by confocal microscopy at a magnification of × 40.

Statistical Analysis

The results were expressed as mean ± SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) or a two-way ANOVA, followed by Dunnett's, Tukey's, or LSD *post hoc* test for comparing the treated group with the control group and multigroup comparisons, respectively. Statistical differences between any two groups were determined using Student's *t*-test; $p < 0.05$ was considered to indicate statistically significant difference.

RESULTS

Effect of 5-HT Depletion on the Antidepressant Effect of LY341495, Ketamine, or Paroxetine in the FST

Systemic administration of LY341495 (1 mg/kg i.p.) significantly reduced the immobility time in the FST ($F(3, 28) = 19.87$, $p < 0.001$; Figure 1a). Systemic administration

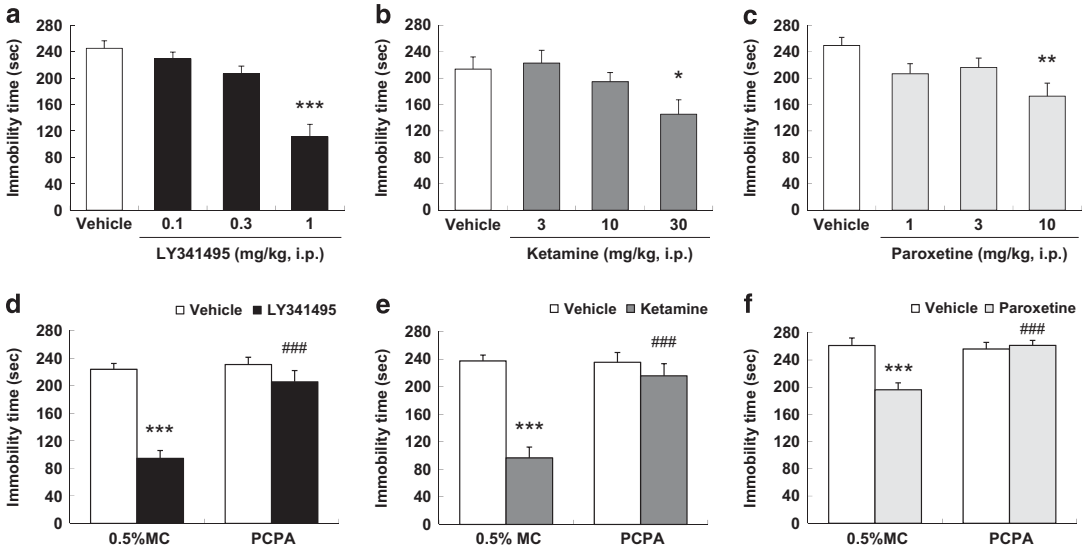

Figure 1 Effect of 5-HT depletion on the antidepressant effect of LY341495, ketamine, or paroxetine in the FST. Effect of LY341495 (a), ketamine (b), or paroxetine (c). LY341495 (0.1, 0.3, and 1 mg/kg i.p.), ketamine (3, 10, and 30 mg/kg i.p.), or paroxetine (1, 3, and 10 mg/kg i.p.) was administered 30 min before the test. Vehicle = (a): 1/15 M phosphate buffer (pH 8.0), (b): saline, (c): distilled water. Values indicate the mean ± SEM ((a): $n = 8$, (b): $n = 8$, (c): $n = 8$). $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with each vehicle (Dunnett's test). Effect of 5-HT depletion on the antidepressant effect of LY341495 (d), ketamine (e), or paroxetine (f). LY341495 (1 mg/kg i.p.), ketamine (30 mg/kg i.p.), or paroxetine (10 mg/kg s.c.) was administered 30 min before the test. PCPA (300 mg/kg i.p.) was administered twice daily for 3 consecutive days until the day before the test. Vehicle = (d): 1/15 M phosphate buffer (pH 8.0), (e): saline, (f): distilled water. Values indicate the mean ± SEM ((d): $n = 10$, (e): $n = 10$, (f): $n = 9$-10). $^{***}P < 0.001$ compared with 0.5% MC-treated each vehicle, $^{###}p < 0.001$ compared with 0.5% MC-treated agents ((d): LY341495, (e): ketamine, (f): paroxetine) (LSD *post hoc* test).

of ketamine (30 mg/kg i.p.) also significantly reduced the immobility time in the FST ($F(3, 28) = 3.45$, $p < 0.05$; Figure 1b). Furthermore, systemic administration of paroxetine (10 mg/kg i.p.) also significantly reduced the immobility time in the FST ($F(3, 28) = 4.15$, $p < 0.05$; Figure 1c). The decrease in the immobility time induced by systemic administration of LY341495 (1 mg/kg i.p.) was blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for 3 days) (LY341495, $F(1, 36) = 41.11$, $p < 0.001$; PCPA, $F(1, 36) = 24.11$, $p < 0.001$; interaction, $F(1, 36) = 18.75$, $p < 0.001$; Figure 1d). Similarly, the decrease in the immobility time induced by systemic administration of ketamine (30 mg/kg i.p.) was also blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for 3 days) (ketamine, $F(1, 36) = 30.69$, $p < 0.001$; PCPA, $F(1, 36) = 16.35$, $p < 0.001$; interaction, $F(1, 36) = 17.43$, $p < 0.001$; Figure 1e). Furthermore, the decrease in the immobility time induced by systemic administration of paroxetine (10 mg/kg s.c.) was also blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for 3 days) (paroxetine, $F(1, 34) = 10.60$, $p < 0.01$; PCPA, $F(1, 34) = 9.52$; $p < 0.01$; interaction, $F(1, 34) = 13.01$, $p < 0.001$; Figure 1f), indicating that the present condition is suitable to investigate the involvement of the 5-HTergic transmission in the antidepressant actions. In contrast, systemic administration of PCPA (300 mg/kg i.p. twice daily for 3 days) *per se* had no effect on the immobility time in the FST (Figure 1d–f). Of note, systemic administration of LY341495 (1, 3 mg/kg i.p.) and ketamine (30 mg/kg i.p.) had no effect on the locomotor activities (LY341495 (1 mg/kg), $F(1, 10) = 2.85$, $p = 0.12$; LY341495 (3 mg/kg), $F(1, 10) = 1.67$, $p = 0.22$; ketamine, $F(1, 10) = 1.71$, $p = 0.22$; Supplementary Table S1).

In treatment with LY341495, ketamine, and paroxetine at 24 h before the test, LY341495 (3.0 mg/kg) and ketamine (30 mg/kg) significantly reduced the immobility time in the FST (LY341495, $F(4, 35) = 3.29$, $p < 0.05$; ketamine, $F(4, 35) = 8.33$, $p < 0.01$; Supplementary Figure S1a and b), whereas paroxetine had no such effect on the immobility time in the FST ($F(3, 28) = 0.10$, $p = 0.96$; Supplementary Figure S1c). The decrease in the immobility time induced by systemic administration of LY341495 (3 mg/kg i.p.) was blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for 3 days) (LY341495, $F(1, 36) = 38.10$, $p < 0.001$; PCPA, $F(1, 36) = 23.15$, $p < 0.001$; interaction, $F(1, 36) = 28.26$, $p < 0.001$; Supplementary Figure S1d). Similarly, the decrease in the immobility time induced by systemic administration of ketamine (30 mg/kg i.p.) was also blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for 3 days) (ketamine, $F(1, 36) = 16.62$, $p < 0.01$; PCPA, $F(1, 36) = 10.09$, $p < 0.001$; interaction, $F(1, 36) = 12.66$, $p < 0.01$; Supplementary Figure S1e). In contrast, administration of PCPA (300 mg/kg i.p. twice daily for 3 days) *per se* had no effect on the immobility time in the FST (Supplementary Figure S1d and e).

Effect of Microinjection of LY341495 or Ketamine into the mPFC in the FST

Microinjection of LY341495 (0.03 pmol/0.1 μl/side) into the mPFC significantly reduced the immobility time in the FST ($F(2, 21) = 14.37$, $p < 0.001$; Figure 2a). Microinjection of ketamine (3 nmol/0.1 μl/side) into the mPFC also significantly reduced the immobility time in the FST ($F(2, 20) = 11.65$, $p < 0.001$; Figure 2b). The decrease in the immobility time induced by microinjection of LY341495 (0.03 pmol/0.1 μl/side) into the mPFC was blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for

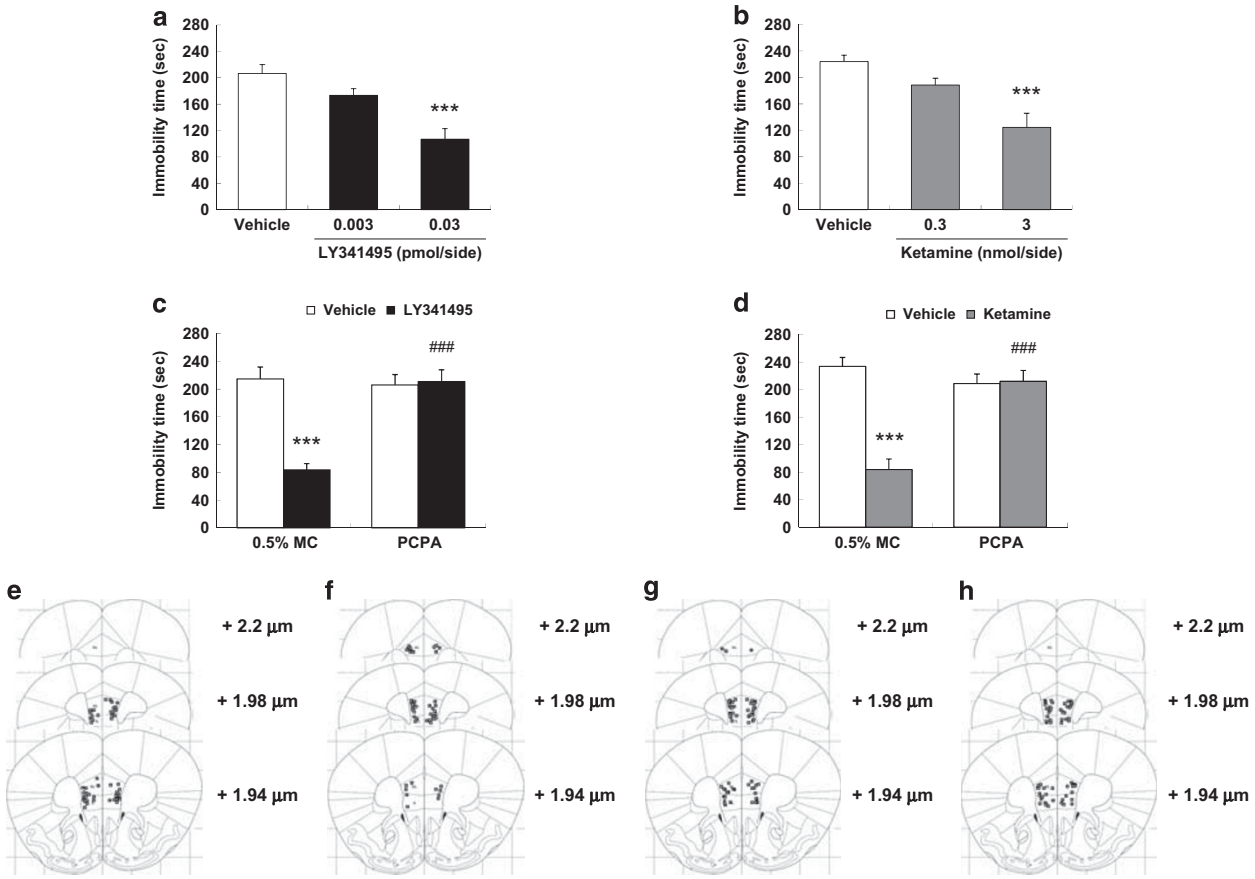

Figure 2 Effect of microinjection of LY341495 or ketamine into the mPFC in the FST. Effect of microinjection of LY341495 (a) or ketamine (b). LY341495 (0.003, and 0.03 pmol/side) or ketamine (0.3 and 3 nmol/side) was administered into the mPFC 30 min before the test. Vehicle = (a): 0.1% 1/15 M phosphate buffer (pH 8.0) in Ringer's solution, (b): Ringer's solution. Values indicate the mean ± SEM ((a): $n = 8$, (b): $n = 7$-8). $^{***}P < 0.001$ compared with each vehicle (Dunnett's test). Effect of 5-HT depletion on the antidepressant effect of microinjection of LY341495 (c) or ketamine (d) into the mPFC. LY341495 (0.03 pmol/side) or ketamine (3 nmol/side) was administered into the mPFC 30 min before the test. PCPA (300 mg/kg i.p.) was administered twice daily for 3 consecutive days until the day before the test. Vehicle = (c): 0.1% 1/15 M phosphate buffer (pH 8.0) in Ringer's solution, (d): Ringer's solution. Values indicate the mean ± SEM ((c): $n = 8$, (d): $n = 7$–8). $^{***}P < 0.001$ compared with 0.5% MC-treated each vehicle, $^{###}p < 0.001$ compared with 0.5% MC-treated agents ((c): LY341495, (d): ketamine) (LSD *post hoc* test). (e, f, g, h) Location of the microinjection cannula tips in the mPFC included in the analyses of the data illustrated ((e) = (a), (f) = (b), (g) = (c), (h) = (d)). The line drawings are from Paxinos and Franklin (1997). The numbers to the right are mm from the bregma. The open square represents the location of the microinjection cannula tips.

3 days) (LY341495, $F(1, 28) = 18.11$, $p < 0.001$; PCPA, $F(1, 28) = 16.02$, $p < 0.001$; interaction, $F(1, 28) = 21.10$, $p < 0.001$; Figure 2c). The decrease in the immobility time induced by microinjection of ketamine (3 nmol/0.1 µl/side) into the mPFC was also blocked by pretreatment with PCPA (300 mg/kg i.p. twice daily for 3 days) (ketamine, $F(1, 27) = 27.03$, $p < 0.001$; PCPA, $F(1, 27) = 13.78$, $p < 0.001$; interaction, $F(1, 27) = 27.45$, $p < 0.001$; Figure 2d). In contrast, PCPA injection (300 mg/kg i.p. twice daily for 3 days) *per se* had no effect on the immobility time in the FST (Figure 2c and d). Of note, microinjection of LY341495 (0.03 pmol/0.1 µl/side) or ketamine (3 nmol/0.1 µl/side) into the mPFC had no effect on the locomotor activities (LY341495, $F(1, 10) < 0.01$, $p = 0.97$; ketamine, $F(1, 10) < 0.01$, $p = 0.96$; Supplementary Table S1).

We further investigated whether the microinjection of LY341495 and ketamine into the mPFC shows sustained antidepressant effects in the FST. The microinjection of LY341495 (0.03 pmol/0.1 µl/side) or ketamine (3 nmol/0.1 µl/side) into the mPFC at 24 h before the test significantly reduced the immobility time in the FST (LY341495, $F(1, 13) = 83.88$, $p < 0.001$; ketamine, $F(1, 14) = 51.64$, $p < 0.001$; Supplementary Figure S2a and b). Figure 2e–h and Supplementary Figure S2c and d show the locations of the tips of the microinjection cannulas in the mPFC included in the analysis of the data shown in Figure 2a–d and Supplementary Figures S2a and b, respectively, that were included in the statistical analyses.

Effect of an AMPA Receptor Antagonist on the Antidepressant Effect of LY341495 or Ketamine in the FST

The decrease in immobility time induced by systemic administration of LY341495 (1 mg/kg i.p.) was blocked by systemic administration of an AMPA receptor antagonist, NBQX (10 mg/kg s.c.) ($F(4, 35) = 13.14$, $p < 0.001$; Figure 3a). Similarly, the decrease in the immobility time induced by systemic administration of ketamine (30 mg/kg i.p.) was also blocked by systemic administration

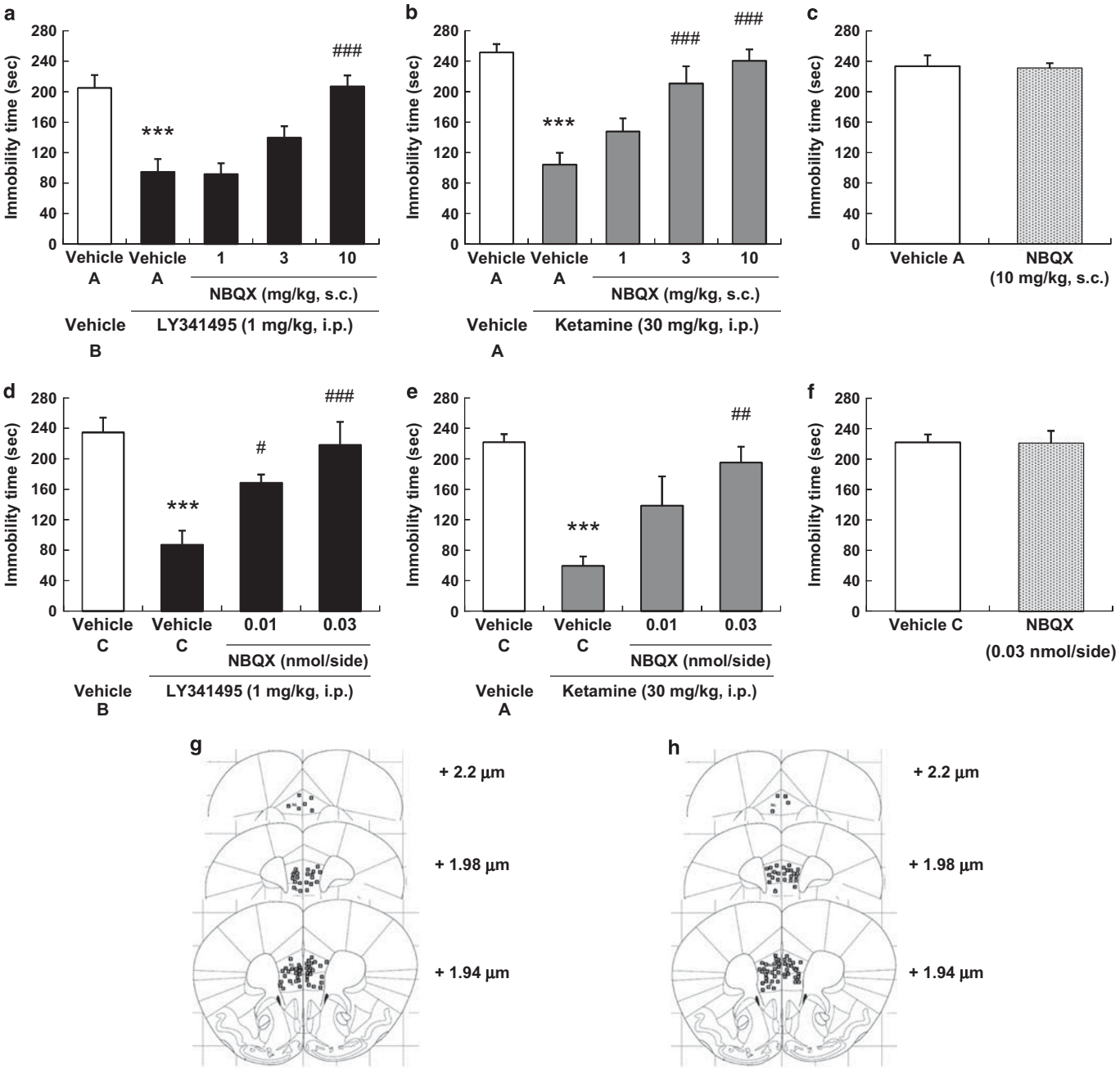

Figure 3 Effect of an AMPA receptor antagonist on the antidepressant effect of LY341495 or ketamine in the FST. Effect of systemic administration of NBQX. (a) LY341495 (1 mg/kg i.p.) or (b) ketamine (30 mg/kg i.p.) was administered 30 min before the test, and NBQX (1, 3, and 10 mg/kg s.c.) was administered 35 min before the test. (c) NBQX (10 mg/kg s.c.) was administered 35 min before the test. Vehicle A: saline, Vehicle B: 1/15 M phosphate buffer (pH 8.0). Values indicate the mean ± SEM ((a): $n=8$, (b): $n=7$–9, (c): $n=8$). $^{***}P<0.001$ compared with vehicle A-treated vehicles ((a): vehicle B, (b): vehicle A), $^{\#\#\#}p<0.001$ compared with vehicle A-treated agents ((a): LY341495, (b): ketamine) (Tukey's *post hoc* test). Effect of microinjection of NBQX into the mPFC. (d) LY341495 (1 mg/kg i.p.) or (e) ketamine (30 mg/kg i.p.) was administered 30 min before the test, and NBQX (0.01 and 0.03 nmol/side) was administered into the mPFC 35 min before the test. (f) NBQX (0.03 nmol/side) was administered into the mPFC 35 min before the test. Vehicle A: saline, Vehicle B: 1/15 M phosphate buffer (pH 8.0), Vehicle C: 10% DMSO in Ringer's solution. Values indicate the mean ± SEM ((d): $n=8$–9, (e): $n=7$–8, (f): $n=7$–8). (g, h) Location of the microinjection cannula tips in the mPFC included in the analyses of the data illustrated ((g) = (d), (h) = (e), (f)). The line drawings are from Paxinos and Franklin (1997). The numbers to the right are mm from the bregma. The open square represents the location of the microinjection cannula tips. $^{***}P<0.001$ compared with vehicle C-treated vehicles ((d): vehicle B, (e): vehicle A), $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with vehicle C-treated agents ((d): LY341495, (e): ketamine) (Tukey's *post hoc* test).

of NBQX (3, 10 mg/kg s.c.) ($F(4, 35)=14.57$, $p<0.001$; Figure 3b). In contrast, systemic administration of NBQX (10 mg/kg s.c.) had no effect on the immobility time in the FST ($F(1, 14)=0.03$, $p=0.88$; Figure 3c) and the locomotor activities ($F(1, 10)<0.01$, $p=0.97$; Supplementary Table S1).

The decrease in the immobility time induced by systemic administration of LY341495 (1 mg/kg i.p.) was blocked by

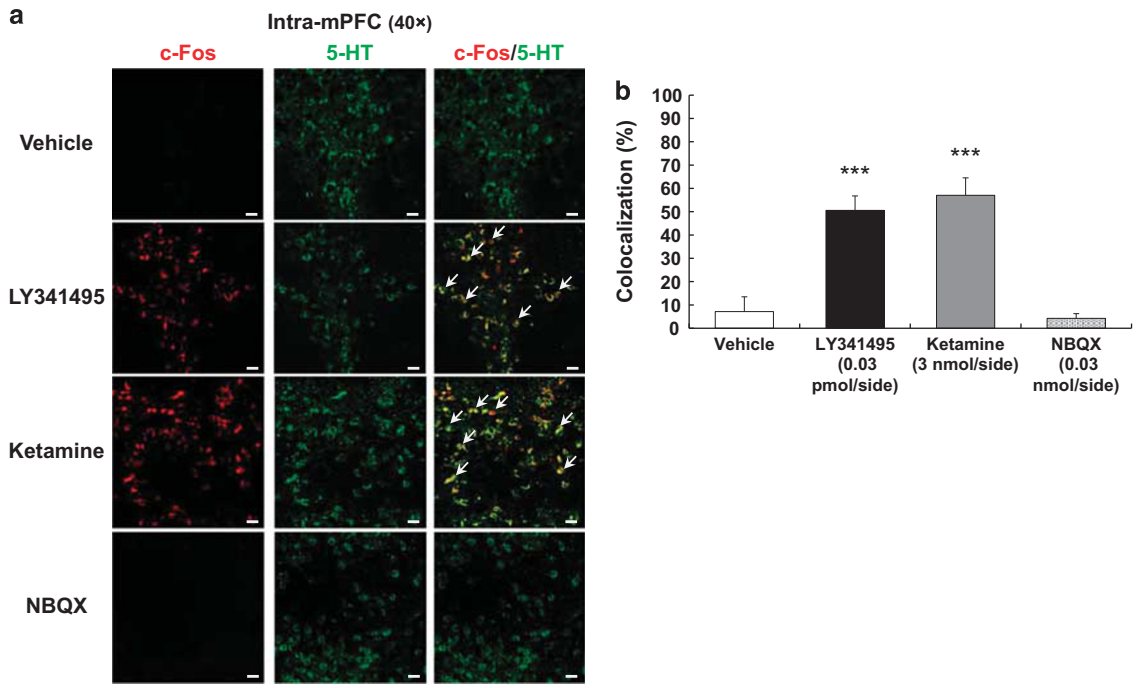

Figure 4 Effect of microinjection of LY341495 or ketamine on the c-Fos immunoreactivity colocalized with 5-HT neuron cells in the dorsal raphe nucleus. (a) Confocal images of c-Fos (red), 5-HT cells (green), and double (5-HT/c-Fos, colocalization) immunoreactivities in the DRN at around − 4.72 mm from bregma (Scale bar: (a) 20 μm). Arrows represent the double (5-HT/c-Fos, colocalization) immunoreactivities. (b) Percentages of c-Fos/5-HT colocalization in the DRN at around − 4.72 mm from bregma. LY341495 (0.03 pmol/side) or ketamine (3 nmol/side) was administered into the mPFC 90 min before the test. NBQX (0.03 nmol/side) was administered into the mPFC 95 min before the test. No differences in the number of single 5-HT-positive cells in the DRN were observed between groups ((b): $n = 43.8 \pm 2.9$ (vehicle), 53.8 ± 9.8 (LY341495), 46.8 ± 5.5 (ketamine), 58.7 ± 9.1 (NBQX)). Vehicle: Ringer's solution. Values indicate the mean ± SEM ($n = 6$). ***$P < 0.001$ compared with vehicle (Student's *t*-test).

microinjection of NBQX (0.03 nmol/0.1 μl/side) into the mPFC ($F(3, 30) = 10.67$, $p < 0.001$; Figure 3d). Similarly, the decrease in the immobility time induced by systemic administration of ketamine (30 mg/kg i.p.) was also blocked by microinjection of NBQX (0.03 nmol/0.1 μl/side) into the mPFC ($F(3, 26) = 10.04$, $p < 0.01$; Figure 3e). In contrast, microinjection of NBQX (0.03 nmol/0.1 μl/side) into the mPFC *per se* had no effect on the immobility time in the FST ($F(1, 13) < 0.01$, $p = 0.96$; Figure 3f). Figure 3g and h show the locations of the tips of the microinjection cannulas in the mPFC included in the analysis of the data shown in Figure 3d–f, respectively, that were included in the statistical analyses.

Effect of Microinjection of LY341495 or Ketamine into the mPFC on the c-Fos Immunoreactivity of 5-HT Neurons in the DRN

Microinjection of LY341494 (0.03 pmol/0.1 μl/side) into the mPFC significantly increased the percentage of 5-HT cell bodies in the DRN exhibiting c-Fos immunoreactivity ($F(1, 10) = 24.01$, $p < 0.001$; Figure 4a and b and Supplementary Figure S4). Microinjection of ketamine (3 nmol/0.1 μl/side) into the mPFC also significantly increased the percentage of 5-HT cell bodies in the DRN showing c-Fos immunoreactivity ($F(1, 10) = 25.80$, $p < 0.001$; Figure 4a and b and Supplementary Figure S4). In contrast, microinjection of NBQX (0.03 nmol/0.1 μl/side) into the mPFC had no effect on the percentage of 5-HT cell bodies in the DRN showing c-Fos immunoreactivity ($F(1, 10) = 0.18$, $p = 0.68$; Figure 4a and b and Supplementary Figure S4). These results are summarized in Supplementary Table S2.

Effect of Microinjection of an AMPA Receptor Antagonist on LY341495 or Ketamine-Induced Increase of the c-Fos Immunoreactivity of 5-HT Neurons in the DRN

Systemic administration of LY341494 (1 mg/kg i.p.) significantly increased the percentage of 5-HT cell bodies in the DRN exhibiting c-Fos immunoreactivity ($F(2, 15) = 17.97$, $p < 0.001$; Figure 5a and c and Supplementary Figure S5a). This increase in c-Fos immunoreactivity in the 5-HT neurons was blocked by prior microinjection of NBQX (0.03 nmol/0.1 μl/side) into the mPFC ($F(2, 15) = 17.97$, $p < 0.001$; Figure 5a and c and Supplementary Figure S5a). Systemic ketamine administration (30 mg/kg i.p.) also significantly increased the percentage of 5-HT cell bodies in the DRN exhibiting c-Fos immunoreactivity ($F(2, 15) = 10.64$, $p < 0.01$; Figure 5b and d and Supplementary Figure S5b). This increase in the percentage of 5-HT cell bodies in the DRN exhibiting c-Fos immunoreactivity was blocked by prior microinjection of NBQX (0.03 nmol/0.1 μl/side) into the mPFC ($F(2, 15) = 10.64$, $p < 0.01$; Figure 5b and d,Supplementary Figure S5b). These results are summarized in Supplementary Table S2.

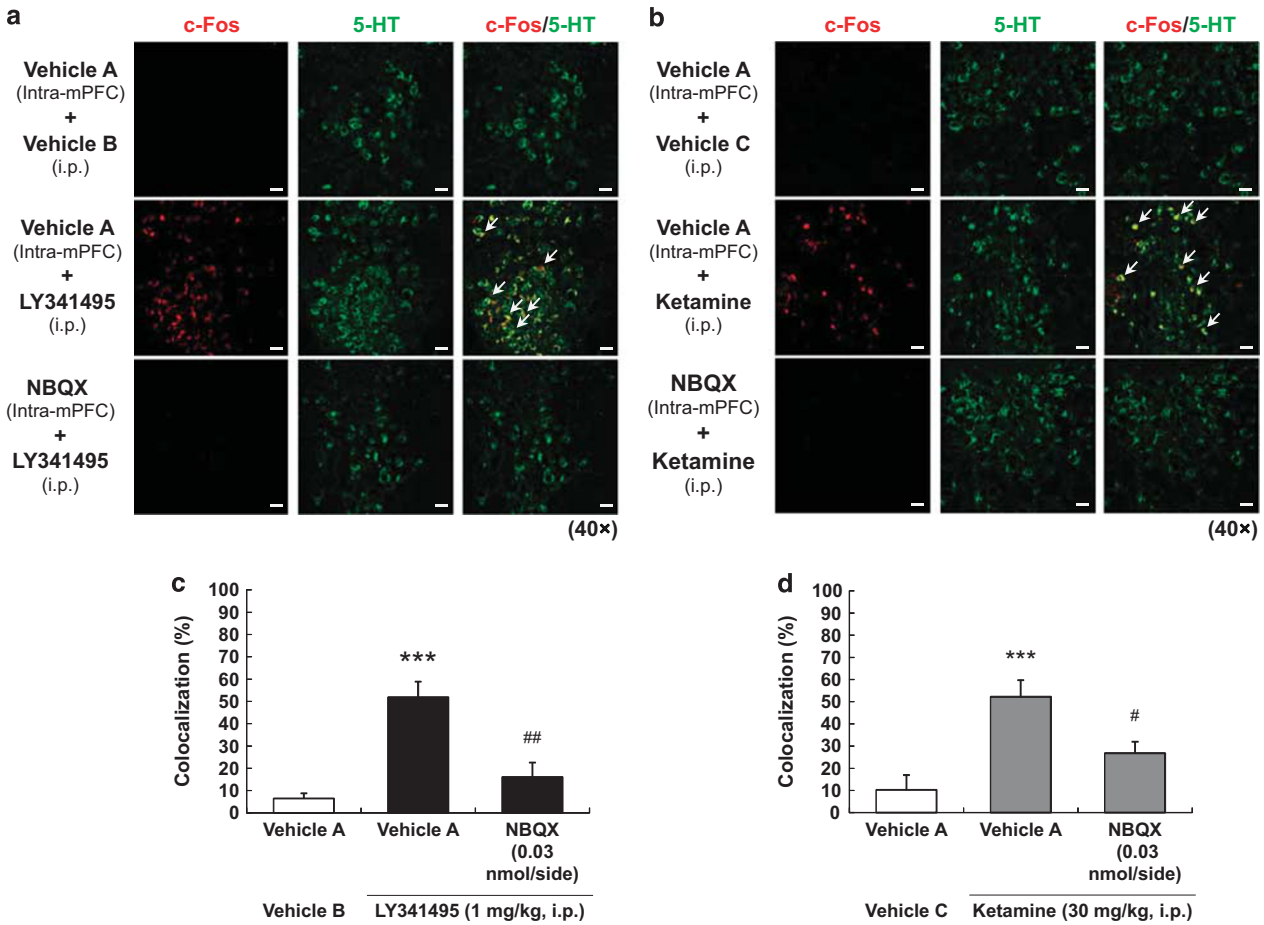

Figure 5 Effect of microinjection of an AMPA receptor antagonist on LY341495- or ketamine-induced c-Fos immunoreactivity colocalized with 5-HT neuron cells in the dorsal raphe nucleus. (a, b) Confocal images of c-Fos (red), 5-HT cells (green), and double (5-HT/c-Fos, colocalization) immunoreactivities in the DRN at around −4.72 mm from bregma (Scale bar: (a, b) 20 μm). Arrows represent the double (5-HT/c-Fos, colocalization) immunoreactivities. (c, d) Percentages of c-Fos/5-HT colocalization in the DRN at around −4.72 mm from bregma following LY341495 (c) or ketamine (d) administration. LY341495 (1 mg/kg i.p.) or ketamine (30 mg/kg, i.p.) was administered 90 min before the test. NBQX (0.03 nmol/side) was administered into the mPFC 95 min before the test. No differences in the number of single 5-HT-positive cells in the DRN were observed between groups ((c): $n=59.5\pm10.1$ (vehicle A-treated vehicle B), 81.3 ± 17.7 (vehicle A-treated LY341495), 67.5 ± 11.3 (NBQX-treated LY341495); (d): $n=84.2\pm8.3$ (vehicle A-treated vehicle C), 91.7 ± 15.1 (vehicle A-treated ketamine), 67.2 ± 9.8 (NBQX-treated ketamine)). Vehicle A: 10% DMSO in Ringer's solution, Vehicle B: 1/15 M phosphate buffer (pH 8.0), Vehicle C: saline. Values indicate the mean ± SEM ((c): $n=6$, (d): $n=6$). $^{***}P<0.001$ compared with vehicle A-treated each vehicles ((c): vehicle B, (d): vehicle C), $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with vehicle A-treated agents ((c): LY341495, (d): ketamine) (Tukey's *post hoc* test).

DISCUSSION

In the present study, we demonstrated, for the first time, that an mGlu2/3 receptor antagonist and ketamine activated a subset of 5-HT neurons in the DRN, presumably through AMPA receptor stimulation in the mPFC, and that this mechanism may be involved in the antidepressant effects of the two compounds.

First, we investigated the involvement of the 5-HTergic systems in the antidepressant effects of an mGlu2/3 receptor antagonist, LY341495, and ketamine in the FST. The antidepressant effects of LY341495 and ketamine were blocked by depletion of 5-HT by pretreatment with a tryptophan hydroxylase inhibitor, PCPA. Previously, we reported that the actions of an mGlu2/3 receptor antagonist and ketamine were attenuated by pretreatment with PCPA in the NSFT (Fukumoto *et al*, 2014). Therefore, the 5-HTergic systems may play a key role in the antidepressant effects of the mGlu2/3 receptor antagonists and ketamine. Moreover, the sustained antidepressant effects of LY341495 and ketamine, which were observed even at 24 h after the treatment, were also attenuated by pretreatment with PCPA, suggesting that the 5-HTergic systems may also be involved in the sustained antidepressant effects of the mGlu2/3 receptor antagonist and ketamine in the FST. On the other hand, paroxetine did not exert the sustained antidepressant effect, suggesting that the mode of action on 5-HTergic systems is different between the mGlu2/3 receptor antagonist and ketamine and paroxetine. Of note, we have confirmed previously that the present treatment condition is sufficient for the pharmacological depletion of 5-HT in mice (Fukumoto *et al*, 2014). Contrary to our findings, it has been reported that the acute antidepressant effects of ketamine and an mGlu2/3 receptor antagonist in the FST and tail suspension test, respectively, were independent of the 5-HTergic systems (Gigliucci *et al*, 2013;

Pałucha-Poniewiera *et al*, 2010). Although these differences may be ascribed to differences in the study paradigms or conditions among the studies, the precise reason remains unclear at present.

An mGlu2/3 receptor antagonist and ketamine have been reported to exert antidepressant effects via their actions on the mPFC (Dwyer *et al*, 2012; Li *et al*, 2010), where both compounds increase synaptic protein synthesis through BDNF–mTOR signaling pathways. Therefore, we investigated the effects of microinjection of LY341495 and ketamine into the mPFC in the FST, and the involvement of the 5-HTergic systems in these effects. In this study, microinjection of LY341495 and ketamine into the mPFC exhibited antidepressant effects in the FST without affecting the locomotor activities, and these effects were attenuated by pretreatment with PCPA. Therefore, the antidepressant effects of the mGlu2/3 receptor antagonist and ketamine appeared to be exerted by modulation of the 5-HTergic neurons through their actions on the mPFC. Moreover, the antidepressant effects of microinjection of LY341495 and ketamine into the mPFC lasted for 24 h after the treatment in the FST, indicating that actions of these compounds in the mPFC are involved in the sustained antidepressant effects.

Next, we investigated the neural mechanisms by which the two compounds regulate the 5-HTergic systems through their actions on the mPFC to exert antidepressant effects. The DRN contains the majority of the 5-HT neurons in the brain that are regulated by neural projections from various brain regions, including the PFC (Pollak Dorocic *et al*, 2014). Stimulation of the AMPA receptor expressed on the pyramidal neurons in the mPFC projecting to the DRN has been reported to activate the 5-HT neurons in the DRN (Martín-Ruiz *et al*, 2001), and AMPA receptor stimulation has been shown to be involved in the antidepressant effects as well as increase in 5-HT release in the mPFC by both mGlu2/3 receptor antagonists and ketamine (Karasawa *et al*, 2005; Koike *et al*, 2011a, b; Maeng *et al*, 2008; Nishitani *et al*, 2014). Given that LY341495 and ketamine reportedly increase the release of glutamate in the PFC, presumably through inhibition of the autoreceptor in the glutamatergic nerve terminals for the case of LY341495 (Hascup *et al*, 2010) and disinhibition of pyramidal neurons in the PFC for the case of ketamine (Moghaddam *et al*, 1997), it is conceivable that both the mGlu2/3 receptor antagonist and ketamine activate postsynaptic AMPA receptor in the mPFC through enhancing the release of glutamate that may lead to subsequent activation of the 5-HTergic systems to exert antidepressant effects. To verify this hypothesis, we first investigated the involvement of the AMPA receptor in the mPFC in the antidepressant effects of the mGlu2/3 receptor antagonist and ketamine. We confirmed that the antidepressant effects of LY341495 and ketamine were abolished by pretreatment with systemic administration of an AMPA receptor antagonist, NBQX, in the present condition as previously reported (Koike *et al*, 2011a, b; Maeng *et al*, 2008; Pałucha-Poniewiera *et al*, 2010). Moreover, the antidepressant effects of LY341495 and ketamine were also blocked by microinjection of NBQX into the mPFC. Therefore, it can be concluded that stimulation of the AMPA receptor in the mPFC is involved in the antidepressant effects of the mGlu2/3 receptor antagonist and ketamine in the FST.

Then, we employed immunohistochemistry to determine whether the antidepressant effects of LY341495 and ketamine mediated by activation of the AMPA receptor in the mPFC may also be associated with activation of the 5-HT neurons in the DRN. Microinjection of LY341495 and ketamine into the mPFC increased c-Fos immunoreactivity in the 5-HT neurons in the DRN. These results suggest that the mGlu2/3 receptor antagonist and ketamine activated the 5-HT neurons in the DRN through their actions in the mPFC. Furthermore, systemic administration of LY341495 and ketamine also increased c-Fos immunoreactivity in the 5-HT neurons in the DRN that were blocked by microinjection of NBQX into the mPFC. Therefore, it was concluded that the mGlu2/3 receptor antagonist and ketamine induced activation of the 5-HT neurons in the DRN through stimulation of AMPA receptor in the mPFC. Taken together, the findings suggest that activation of a subset of 5-HT neurons in the DRN modulated by stimulation of the AMPA receptor in the mPFC may be involved in the antidepressant effects of the mGlu2/3 receptor antagonist and ketamine. Some previous reports also lend support to this notion. Recently, it was reported that antidepressant effects could be obtained in the rats by selective stimulation, using an optogenetic technique, of the neurons in the DRN regulated by mPFC projections, whereas no such effect was obtained following activation of the entire population of DRN neurons (Warden *et al*, 2012). Another report indicated that DBS of the mPFC yielded antidepressant effects, which was abolished by 5-HT depletion (Hamani *et al*, 2010). These reports suggest that 5-HTergic systems selectively modulated by mPFC–DRN projections may be involved in the antidepressant effects. In contrast, a previous study reported that excitatory ventromedial PFC (vmPFC) projections were primarily localized in the GABA-rich areas of the DRN, and that optogenetic activation of the vmPFC terminals in the DRN increased c-Fos expression in the GABA neurons to a great extent than that in the 5-HT neurons (Challis *et al*, 2014), consistent with the previously reported findings that the mPFC projections to the DRN preferentially target local-circuit GABAergic neurons (Jankowski and Sesack, 2004; Varga *et al*, 2003; Wang *et al*, 1992). These differences may be because of the differences in the population of neurons in the DRN modulated by mPFC projections. Recently, identification of monosynaptic glutamatergic inputs from the PFC to 5-HTergic neurons in the DRN was reported (Pollak Dorocic *et al*, 2014), indicating that the direct PFC–DRN pathway that exerts excitatory control over 5-HTergic neurons in the DRN exists. Moreover, the other group has reported that the PFC preferentially innervates the 5-HT neurons over the GABA neurons in the DRN, and that cortical axon photoactivation was twice as efficient at triggering EPSCs in the 5-HT neurons than in the GABA neurons (Weissbourd *et al*, 2014), indicating, as observed in the present study, that a part of the mPFC projections may preferentially activate the 5-HT neurons in the DRN. It should be noted, however, that one of the limitations of the present study is that we used only FST, and the present findings should be confirmed in other animal models of depression in future studies.

In conclusion, we provide evidence, for the first time, that the antidepressant effects of an mGlu2/3 receptor antagonist and ketamine may be mediated by the activation of a subset

of 5-HT neurons in the DRN modulated by stimulation of the AMPA receptor in the mPFC. Therefore, the present findings provide an understanding of the neural mechanisms underlying the antidepressant effects of the mGlu2/3 receptor antagonists and ketamine, and further investigation may pave the way for innovative therapies for the treatment of major depressive disorder. Moreover, given that mGlu2/3 receptor antagonists share the similar mechanisms of action with ketamine, yet they did not show undesirable side effects in humans (Gradient *et al*, 2012), mGlu2/3 receptor antagonists could be alternatives to ketamine.

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The authors declare no conflict of interest. Kenichi Fukumoto, Michihiko Iijima, and Shigeyuki Chaki are employees of Taisho Pharmaceutical.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)