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Blonanserin ameliorates social deficit through dopamine-D_3 receptor antagonism in mice administered phencyclidine as an animal model of schizophrenia

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ABSTRACT

Blonanserin differs from other antipsychotic drugs, such as risperidone and olanzapine, and exhibits a higher affinity for dopamine-$D_{2/3}$ receptors than for serotonin 5-HT_{2A} receptors. We investigated the involvement of dopamine-D_3 receptors in the effect of blonanserin on the social deficit observed in an animal model of schizophrenia and sought to elucidate the molecular mechanism underlying its action. Mice received phencyclidine (PCP: 10 mg/kg/day, s.c.), a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, once a day for 14 consecutive days. We then evaluated the sociability, using a social interaction test, and the expression of GluN1 subunit, an essential subunit of the NMDA receptors, in these mice. Blonanserin significantly ameliorated the PCP-induced social deficit, whereas olanzapine and haloperidol did not. This effect of blonanserin was antagonized by 7-OH-DPAT, a dopamine-D_3 receptor agonist, and SCH23390, a dopamine-D_1 receptor antagonist. However, the ameliorating effect of blonanserin was not inhibited by DOI, a serotonin 5-HT_{2A} receptor agonist. The PCP-induced social deficit was also ameliorated by U99194, a dopamine-D_3 receptor antagonist and SKF38393, a dopamine-D_1 receptor agonist, being effects antagonized by 7-OH-DPAT or SCH23390. Blonanserin significantly inhibited the decrease in the phosphorylation levels of GluN1 at Ser^{897} by protein kinase A (PKA) in the prefrontal cortex (PFC) in PCP—administered mice. These results suggest that activation of NMDA receptors due to Ser^{897}-phosphorylation of GluN1 subunit, which is a step linked to dopamine-D_1 receptor-PKA signaling through dopamine-D_3 receptor antagonism in the PFC, is required for the ameliorating effect of blonanserin on the PCP-induced social deficit. These findings also provide *in vivo* evidence that blonanserin antagonism of the dopamine-D_3 receptors may be useful as a novel treatment strategy and that the dopamine-D_3 receptors can be a novel therapeutic target molecule for the social deficit observed in schizophrenia.

1. Introduction

The negative symptoms that are characteristic of schizophrenia include apathy, social deficit, and poverty of thinking (Kibel et al., 1993). Social deficit, such as social withdrawal, is the first sign, and a key component, of the negative symptoms of schizophrenia. Social deficit presents as an inability to engage in conservation, behavior, or work appropriately in daily and social life (Hansen et al., 2009). Because a social deficit also tends to be misinterpreted as laziness or social incompatibility, it is more difficult to identify or understand than the positive symptoms of schizophrenia or cognitive impairments (Penn et al., 1994; Pallanti et al., 2004; Achim et al., 2013). Both negative symptoms and cognitive impairments are strongly associated with social functioning, independent living skills, and the quality of life. Thus,

Abbreviations: ANOVA, analysis of variance; APD, antipsychotic drug; DOI, R(−)-2,5-dimethoxy-4-iodoamphetamine; mPFC, medial prefrontal cortex; NMDA, *N*-methyl-D-aspartate; 7-OH-DPAT, (±)-7-hydroxy-N,N-di-n-propyl-2-aminotetralin; PKA, protein kinase A; PCP, phencyclidine; PVDF, polyvinylidene difluoride; SCH23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SDS, sodium dodecyl sulfate; SKF38393, (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol; U99194, 2,3-dihydro-5,6-dimethoxy-N, N-dipropyl-1H-inden-2-amine

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there is an urgent need to develop novel pharmacotherapies that can effectively treat these dysfunctions (Potasiewicz et al., 2017).

Amisulpride, a benzamide derivative, has high and similar affinities for the dopamine-D_2 and D_3 receptors and is devoid of any significant affinity for other receptors (Schoemaker et al., 1997; Natesan et al., 2008). Amisulpride shows all the attributes of atypical antipsychotic drugs (APDs): lower risk of extrapyramidal symptoms, a somewhat greater improvement in positive and negative symptoms including the social impairment, and better overall outcome in longer term follow up studies compared to some serotonin-dopamine or multi-receptor atypical APDs (Leucht et al., 2002; Martin et al., 2002; Sechter et al., 2002; Davis et al., 2003; Natesan et al., 2008). Thus, amisulpride provides a unique vantage, it is an atypical APD and yet unlike other atypical ADPs that bind to multiple receptors, and it only binds to the dopamine-$D_{2/3}$ receptors. Blonanserin, unlike most atypical APDs, has a slightly higher affinity for dopamine-D_2 than serotonin 5-HT_{2A} receptors (Une and Kurumiya, 2007; Ohoyama et al., 2011; Huang et al., 2015), indicating approximate equivalence, unlike the marked differences favoring serotonin 5-HT_{2A} receptors in other atypical APDs (Meltzer and Huang, 2008; Ohoyama et al., 2011). Clinically, blonanserin exhibits atypical APD properties, efficiently treating both positive and negative symptoms as well as the cognitive impairments observed in schizophrenia (Tenjin et al., 2013). The potential tolerability benefits of this drug, in short-term trials on patients with schizophrenia, include fewer extrapyramidal symptoms than those observed in response to haloperidol (Garcia et al., 2009), and fewer reports of prolactin-level increases (hyperprolactinemia) than those observed in response to risperidone (Takahashi et al., 2013). Blonanserin is also generally well tolerated and appears to have an acceptable profile in terms of body-weight gain. Blonanserin, as well as amisulpride, is also a potent antagonist for dopamine-D_2 and D_3 receptors (Une and Kurumiya, 2007; Tadori et al., 2011; Takaki and Ujike, 2013; Huang et al., 2015). Blonanserin has been reported to extensively occupy rat dopamine-D_3 receptors at dosages producing plasma levels equivalent to antipsychotic doses in man (Baba et al., 2015; Huang et al., 2015).

In animal studies, blonanserin has been shown to successfully reduce several psychobehavioral abnormalities in mice administered phencyclidine (PCP), a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist that induces schizophrenia-like psychotomimetic states in humans and rodents (Javitt and Zukin, 1991; Noda et al., 1995, 2001; Castner et al., 2004; Morris et al., 2005). PCP-induced hyperlocomotion, which is considered a model of mesolimbic dopaminergic hyperfunctions of schizophrenia, is significantly antagonized by blonanserin (Jentsch et al., 1998; Nagai et al., 2003; Mouri et al., 2007a). The potentiation of forced swimming-induced immobility following PCP administration, which is considered a model of mesocortical dopaminergic hypofunctions of schizophrenia, is also antagonized by blonanserin, and other atypical APDs (Noda et al., 2000; Nagai et al., 2003). Recently, blonanserin, but not other APDs, such as olanzapine, haloperidol, and risperidone, was found to extensively occupy dopamine-D_3 receptors *in vivo* (Baba et al., 2015), and reverses PCP-induced cognitive impairment in a novel object recognition test (Hida et al., 2015). Hida et al. demonstrated that this ameliorating effect of blonanserin on PCP-induced cognitive impairment was associated with indirect activation of NMDA receptors due to Ser^{897}-phosphorylation of GluN1 subunit, a step linked to dopamine-D_1 receptor-protein kinase A (PKA) signaling following facilitation of dopamine release *in vivo*. The latter was due to a dual antagonism of serotonin 5-HT_{2A} and dopamine-D_3 receptors by blonanserin (Hida et al., 2015).

Preclinical evidence suggests that dopamine-D_3 receptors influence the social functions (Kagaya et al., 1996). Unlike other atypical APDs, blonanserin has a high affinity for dopamine-D_3 receptors (Tenjin et al., 2013) and enhances the social functions in schizophrenia (Kishi et al., 2013; Hori et al., 2014). However, the effect of blonanserin on the social deficit, as a negative symptom, observed in models of schizophrenia and the involvement of dopamine-D_3 receptors in the effect of blonanserin on the social deficit remains unclear, despite previous basic and clinic studies. The aim of this study was to examine the effect of blonanserin on the social behaviors in mice following PCP administration, and to further elucidate the involvement of dopamine-D_3 receptors in this model.

2. Materials and methods

2.1. Animals

Male mice of the ICR strain were obtained from Japan SLC Inc. (Shizuoka, Japan). Mice were 6 weeks of age at the beginning of the experiments. The mice were housed in plastic cages and maintained in a regulated environment (22 ± 2 °C, 55 ± 10% humidity), with a 12-h light/dark cycle (lights on at 09:00 a.m.). Food (CE2; CLEA Japan, Tokyo, Japan) and tap water were available *ad libitum*.

Behavioral experiments were performed in a sound-attenuated and air-regulated room, to which mice were habituated for at least 1-h prior to recording. Behavioral tests were performed during light periods. All experiments were conducted blind to the experimental group and were performed using the different mice independently in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine (Approved number 29,228) and Meijo University Faculty of Pharmacy (Approved number 2017–16). The procedures involving animals and their care conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 8th edition (NIH Publication, 2011).

2.2. Drugs

Blonanserin was supplied by Sumitomo Dainippon Pharma (Osaka, Japan). Other drugs that were used included: olanzapine (Toronto Research Chemicals, Toronto, Canada), haloperidol (Sigma-Aldrich, St. Louis, MO), 7-OH-DPAT [(±)-7-hydroxy N,N-di-n-propyl-2-aminotetralin hydrobromide; RBI-Funakoshi, Tokyo, Japan], DOI [R (−)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; RBI-Funakoshi], SCH23390 [R (+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; RBI-Funakoshi], SKF38393 [(±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride; RBI-Funakoshi] and U99194 [2,3-dihydro-5,6-dimethoxy-N,N-dipropyl-1H-inden-2-amine maleate; Tocris Bioscience, Bristol, UK]. Phencyclidine hydrochloride [1-(1-phenylcyclohexyl) piperidine hydrochloride: PCP] was synthesized by Professor Shinji Kitagaki (Department of Medicinal Chemistry, Faculty of Pharmacy, Meijo University) according to the method described by Maddox et al. (1965) and was checked for purity.

Blonanserin and olanzapine were initially dissolved in a minimum amount of 1N HCl and diluted with saline. Haloperidol was initially dissolved in a minimum amount of 17.5N acetic acid and diluted with saline. The pH of these solution was about 6.0–6.5. 7-OH-DPAT, DOI, SKF38393, U99194, and PCP were dissolved in saline. SCH23390 was initially dissolved in a minimum amount of distilled water and diluted with saline.

2.3. Drug administrations

Mice received saline or PCP (10 mg/kg/day, s.c.) once a day for 14 consecutive days (Noda et al., 1995). The social interaction test was commenced one day after withdrawal of PCP administration. Saline- or PCP-administered mice were treated with blonanserin (0.3 and 1 mg/kg, p.o.) or olanzapine (0.3 and 1 mg/kg, p.o.), using a disposable feeding needle, 30 min before test session. SKF38393 (6 mg/kg, i.p.) was used as a dopamine-D_1 receptor agonist, haloperidol (0.01 and 0.03 mg/kg, i.p.) was used as a dopamine-D_2 receptor antagonist, and U99194 (5 mg/kg, s.c.) was used as a selective dopamine-D_3 receptor antagonist. These drugs were administered 30, 30, and 20 min,

respectively, before test session. SCH29930 (0.01 mg/kg, s.c.) was used as a dopamine-D_1 receptor antagonist, 7-OH-DPAT (0.05 mg/kg, i.p.) was used as a dopamine-D_3 receptor agonist, and DOI (3 mg/kg, i.p.) was used as a serotonin 5-HT_{2A} receptor agonist. These drugs were administered 60, 60, and 5 min, respectively, before test session.

The doses of blonanserin, olanzapine, DOI, and SKF38393 that were used in the present study were determined according to previous publications (Ninan and Kulkarni, 1999; Nagai et al., 2003; Enomoto et al., 2005; Hida et al., 2015): the doses of 7-OH-DPAT were based on previous reports demonstrating the activation of dopamine-D_3 receptors at low doses and the increased occupancy of the dopamine-D_2 receptors at higher doses (> 0.3 mg/kg; Daly and Waddington, 1993; Ahlenius and Salmi, 1994; Levant et al., 1996; Pritchard et al., 2003). Spontaneous activity was measured following treatment with blonanserin, olanzapine, haloperidol, U99194, or SCH23390, and experiments were performed using doses of these drugs without affecting locomotor activity (Supplemental Fig. S1). SKF38393 at up to 10 mg/kg also has been reported to no change in the motility (Kamei et al., 1995). All compounds were systemically administered at a volume of 0.1 ml/10 g body weight. Control mice received the same volume of the vehicle solution.

2.4. Social interaction test

The social interaction task was performed on days 1–3 after the final injection of PCP, in accordance with a protocol described previously (Hida et al., 2014). The apparatus used for the social interaction test consisted of a square open arena (W26 × D31 × H25 cm) made of gray non-reflecting acrylic, illuminated with lamps that could not be seen directly by the mice. All behavioral tasks were conducted under conditions of dim illumination (20 lux). Before the test, each mouse (including the unfamiliar partner mice) was placed alone in the apparatus for 10 min on two consecutive days (habituation: days 1–2). On the test day (day 3), each test mouse was placed in the test box simultaneously with an unknown test partner. The behaviors of the pairs of unfamiliar mice were videotaped for 10 min. The mice were then returned to their home cages. At the end of the test, any debris were removed from the box and the floor and walls of the box were wiped with detergent and dried. Social behaviors, such as sniffing and grooming the partner, following, mounting, and crawling under or over the partner, were recorded separately. It should be emphasized that passive contact (sitting or lying with their bodies in contact) was not included in the social interaction score. The unknown test partner mice that were used were not treated with any compounds.

2.5. Western blotting

Western blotting was performed as previously described, with a minor modification (Hida et al., 2014). The mice were sacrificed by decapitation immediately after the social interaction test, and the brain was immediately removed. The PFC, containing the cingulate and prelimbic area (Bregma +2.96 to +1.34), was defined according to a mouse brain atlas by Paxinos and Franklin (1996). The PFC was rapidly dissected out, frozen, and stored at −80 °C until analysis. To prepare the extracted tissue, the dissected brain tissue was homogenized by sonication in an ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate] supplemented with a mixture of proteinase inhibitors (CompleteTM, Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at 16,000 × *g* for 20 min and the supernatant was used. The protein concentration was determined using a DC Protein Assay Kit (Bio-rad, Richmond, CA, USA). Samples (10–100 μg of protein) were boiled in the sample buffer (125 mM Tris–HCl pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue), separated on a polyacrylamide gel, and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, MA, USA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, MD, USA) and probed with a primary antibody. Membranes were washed with a pH 7.4 washing buffer (50 mM Tris–HCl, 0.05% Tween 20, and 150 mM NaCl) and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. The immune complexes were detected by EZ capture MG (ATTO, Tokyo, Japan) based on chemiluminescence (Chemi-Lumi One Ultra, Nacalai Tesque, Kyoto, Japan). The band intensities were analyzed by densitometry using the ATTO Densitograph Software Library Lane Analyzer (ATTO). To confirm equal loading of each protein, membranes were stripped with a WB Stripping Solution Strong (Nacalai Tesque) for 15 min, and β-actin protein expression was detected as described above. The primary antibodies that were used included: rabbit anti-phospho-GluN1 (Ser^{897}) (1:1000; Millipore Corporation), rabbit anti-phospho-GluN1 (Ser^{896}) (1:1000; Upstate Biotechnology, NY, USA), rabbit anti-GluN1 (1:1000; Santa Cruz Biotechnology, CA, USA), and goat anti-β-actin (1:500; Santa Cruz Biotechnology) antibodies. The secondary antibodies, used at a dilution of 1:2000, were horseradish peroxidase-linked anti-rabbit or anti-goat IgG (Kirkegaard and Perry Laboratories). The amount of total GluN1 loaded was normalized using an antibody against β-actin. MagicMark™ XP Western Protein Standard (Invitrogen, CA, USA) and Precision Plus Protein™ Dual Color Standards (Bio-rad) were used as molecular weight markers. To evaluate GluN1 activation, phosphorylated GluN1 levels were normalized to the total GluN1 level on the same membranes, and then, each phosphorylated/total level was normalized to the basal phosphorylated/total level of saline control mice.

2.6. Statistical analysis

All results are expressed as the mean ± S.E.M. for each group. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's test for multigroup comparisons. The Student's *t*-test was used to compare two sets of data. $P < 0.05$ was considered to indicate statistically significant differences.

3. Results

3.1. Effect of blonanserin on the social deficit in mice that received repeated PCP administration, in comparison with those treated with olanzapine and haloperidol

We examined whether the PCP-induced social deficit was reversed by blonanserin treatment and compared this with the effect of olanzapine and haloperidol. Three days after PCP withdrawal, mice were subjected to the social interaction test. Animals were treated acutely with blonanserin (0.3 and 1 mg/kg, p.o.), olanzapine (0.3 and 1 mg/kg, p.o.), or haloperidol (0.01 and 0.03 mg/kg, i.p.) 30 min before the test session.

As shown in Fig. 1, the total time spent socially interacting with an unfamiliar mouse in the test sessions was significantly lower in PCP-administered mice than in mice administered saline. This indicates a deficiency in the social behavior. Treatment with blonanserin (1 mg/kg, p.o.) significantly ameliorated the social deficit observed in PCP-administered mice. Treatment with blonanserin (1 mg/kg, p.o.) did not affect the social behaviors in saline-administered mice (Fig. 1A). Conversely, neither olanzapine nor haloperidol affected the social deficit in PCP-administered mice (Fig. 1B and C). In addition, olanzapine decreased the social interaction time in saline-administered mice (Fig. 1B), although there was no difference in the spontaneous activity in a novel environment for the 10 min test period between the any of the groups of mice (Supplemental Fig. S1a).

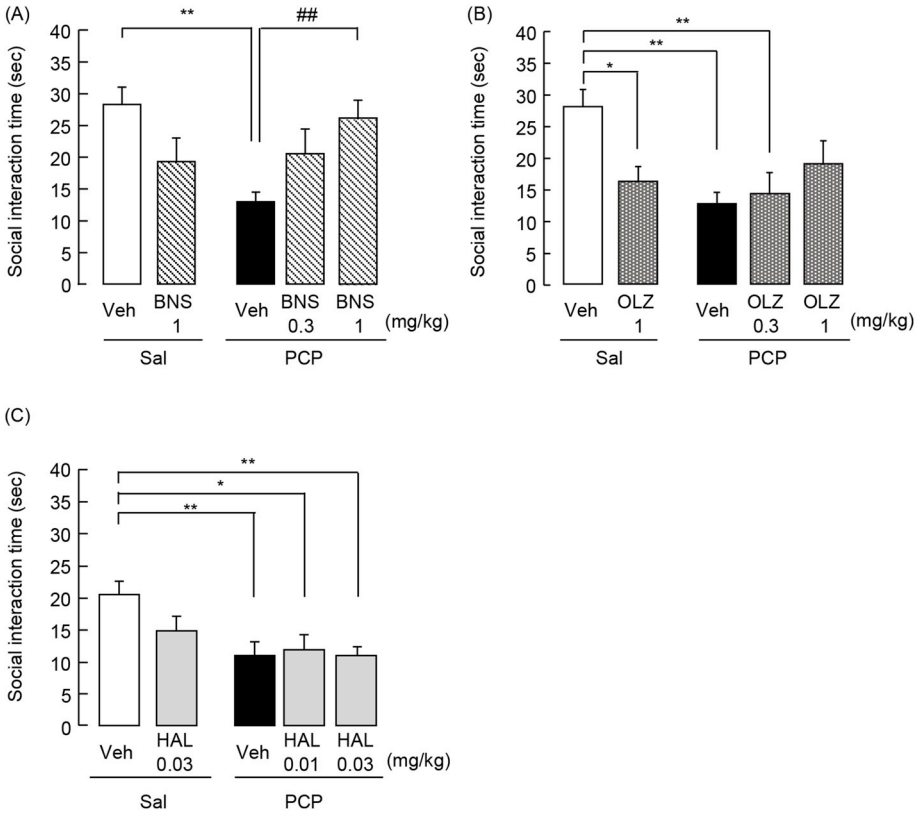

Fig. 1. Effect of blonanserin, olanzapine, and haloperidol on the social behaviors following repeated PCP administration. PCP-administered mice were treated with blonanserin (0.3 and 1 mg/kg, p.o.; A), olanzapine (0.3 and 1 mg/kg, p.o.; B), and haloperidol (0.01 and 0.03 mg/kg, i.p.; C) 30 min before the social interaction test session. Social interaction time was measured over 10-min period. (A) Effect of blonanserin. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 14, (Sal/BNS)-administered mice: n = 11, (PCP/Veh)-administered mice: n = 15, (PCP/BNS 0.3)-administered mice: n = 10, (PCP/BNS 1)-administered mice: n = 13]. One-way ANOVA: $F_{(4,\ 58)} = 5.64$, $p < 0.01$. (B) Effect of olanzapine. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 14, (Sal/OLZ)-administered mice: n = 11, (PCP/Veh)-administered mice: n = 15, (PCP/OLZ 0.3)-administered mice: n = 10, (PCP/OLZ 1)-administered mice: n = 10]. One-way ANOVA: $F_{(4,\ 55)} = 6.73$, $p < 0.01$. (C) Effect of haloperidol. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 11, (Sal/HAL)-administered mice: n = 12, (PCP/Veh)-administered mice: n = 10, (PCP/HAL 0.01)-administered mice: n = 11, (PCP/HAL 0.03)-administered mice: n = 16]. One-way ANOVA: $F_{(4,\ 55)} = 4.67$, $p < 0.01$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs corresponding (Sal/Veh)-administered mice, $^{\#\#}p < 0.01$ vs (PCP/Veh)-administered mice (Tukey's test). Sal: saline, Veh: vehicle, PCP: phencyclidine, BNS: blonanserin, OLZ: olanzapine, HAL: haloperidol.

3.2. Involvement of dopamine-D_3 and serotonin 5-HT_{2A} receptors in the effect of blonanserin on the social behavior of PCP-administered mice

Blonanserin has been demonstrated to have a high affinity not only for the dopamine-D_2 receptors, but also for dopamine-D_3 and serotonin 5-HT_{2A} receptors (Tenjin et al., 2013). To determine whether the ameliorating effect of blonanserin on the PCP-induced social deficit involved dopamine-D_3 or serotonin 5-HT_{2A} receptors, we employed 7-OH-DPAT, a dopamine-D_3 receptor agonist, and DOI, a serotonin 5-HT_{2A} receptor agonist. We tested the effects of these drugs on the amelioration of the PCP-induced social deficit following treatment with blonanserin.

7-OH-DPAT (0.05 mg/kg, i.p.) significantly and completely prevented the ameliorating effect of blonanserin on the social deficit in PCP-administered mice (Fig. 2A), whereas DOI (3 mg/kg, i.p.) administration did not change the effect of blonanserin (Fig. 2B). In saline-administered mice, no significant difference was observed in the social interaction time observed across all groups (Fig. 2A and B).

Next, we investigated whether the PCP-induced social deficit was reversed by U99194, a dopamine-D_3 receptor antagonist. Treatment with U99194 (5 mg/kg, s.c.) significantly ameliorated the social deficit in PCP-administered mice (Fig. 2C). The administration of 7-OH-DPAT (0.05 mg/kg, i.p.) significantly and completely prevented the ameliorating effect of U99194 at a dose of 5 mg/kg (Fig. 2C). In saline-administered mice, there was no significant difference in the social interaction time between the groups treated with U99194 or 7-OH-DPAT (Figs. 1A and 2C).

3.3. Involvement of the dopamine-D_1-NMDA receptor pathway in the effect of blonanserin on the social behavior of PCP-administered mice

Previous studies have shown that the effect of blonanserin on PCP-induced cognitive impairment is associated with indirect functional stimulation of the dopamine-D_1-PKA-NMDA receptor pathway, following augmentation of dopaminergic neurotransmission (Hida et al., 2015). We investigated whether the social amelioration effect of blonanserin was involved in the activation of dopamine-D_1 receptor and NMDA receptor pathways.

SKF38393 (6 mg/kg, i.p.), a dopamine-D_1 receptor agonist, as well as blonanserin, significantly ameliorated the social deficit observed in PCP-administered mice (Fig. 3A and B). The ameliorating effect of both drugs was significantly and completely prevented by SCH23390, a dopamine-D_1 receptor antagonist (Fig. 3A and B). There was no significant difference in the social interaction time across any of the groups in saline-administered mice (Fig. 3A and B).

It is established that PKA phosphorylates GluN1, an essential subunit of the NMDA receptors, at Ser^{897} and regulates its functions (Mouri et al., 2007b). To further examine the mechanism through which blonanserin ameliorates the social deficit in PCP-administered mice, we examined the effect of blonanserin on the Ser^{897}-phosphorylation levels of GluN1 in the PFC of PCP-administered mice immediately after the social interaction test. In the PFC, the Ser^{897}-phosphorylation levels were significantly lower in PCP-administered mice than in the saline-administered mice (Fig. 4A). Treatment with blonanserin (1 mg/kg, p.o.) significantly inhibited the decrease in the levels of Ser^{897}-phosphorylation in PCP-administered mice. However, treatment with olanzapine (1 mg/kg p.o.) did not significantly inhibit the decreased Ser^{897}-phosphorylation levels in PCP-administered mice. There was no significant difference in the phosphorylation levels across any of the groups in saline-administered mice (Fig. 4A, Supplemental Fig. S2A). Furthermore, there was no significant difference in the levels of GluN1 phosphorylation at Ser^{896} (as opposed to Ser^{897}), which is phosphorylated by PKC (as opposed to PKA), in the PFC across any of the groups (Fig. 4B, Supplemental Fig. S2B). No significant group-wise variation was observed in the levels of total GluN1 (Fig. 4C, Supplemental Fig. S2C).

4. Discussion

In this study, we have reconfirmed that PCP-administered mice

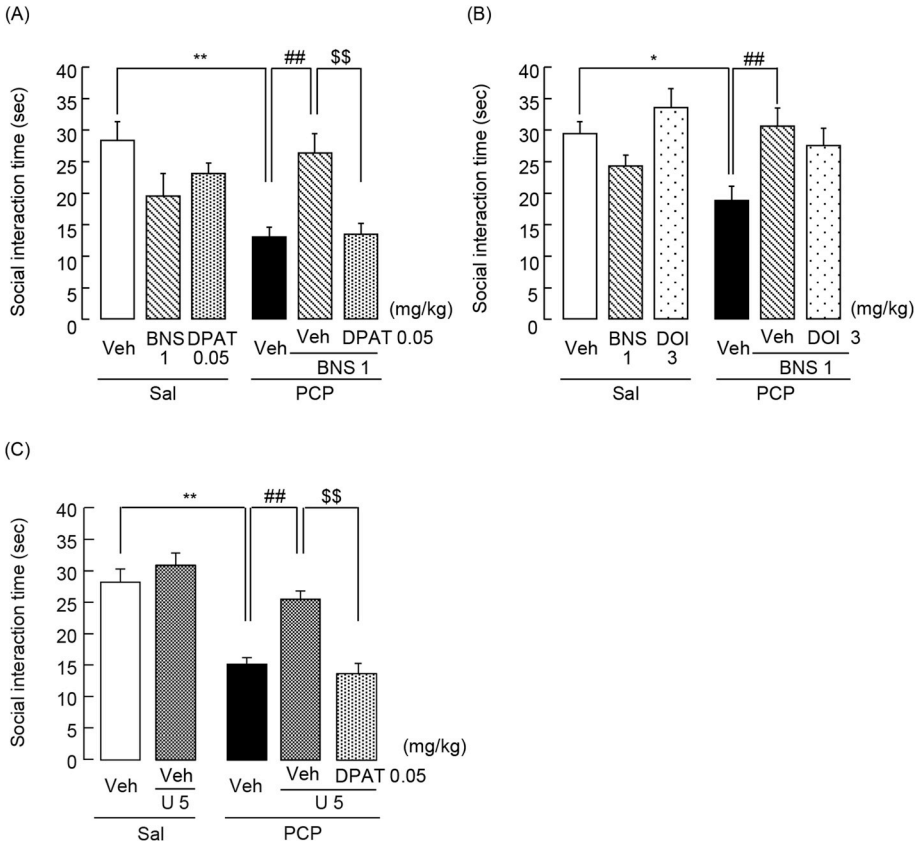

Fig. 2. Involvement of dopamine-D_3 and serotonin 5-HT_{2A} receptors in the social amelioration effect of blonanserin in PCP-administered mice. Mice were administrated with 7-OH-DPAT (0.05 mg/kg, i.p.; A), DOI (3 mg/kg, i.p.; B), or U99194 (5 mg/kg, s.c.; C) 60, 5, or 20 min, respectively, before the social interaction test session. Social interaction time was measured over a 10-min period. (A) Blonanserin + 7-OH-DPAT. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 14, (Sal/BNS 1)-administered mice: n = 11, (Sal/DPAT 0.05)-administered mice: n = 12, (PCP/Veh)-administered mice: n = 15, (PCP/BNS 1/Veh)-administered mice: n = 13, (PCP/BNS 1/DPAT 0.05)-administered mice: n = 16]. One-way ANOVA: $F_{(5, 75)} = 9.03$, $p < 0.01$. (B) Blonanserin + DOI. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 15, (Sal/BNS 1)-administered mice: n = 15, (Sal/DOI 3)-administered mice: n = 15, (PCP/Veh)-administered mice: n = 16, (PCP/BNS 1/Veh)-administered mice: n = 15, (PCP/BNS 1/DOI 3)-administered mice: n = 14]. One-way ANOVA: $F_{(5, 84)} = 5.24$, $p < 0.01$. (C) Effect of U99194. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 21, (Sal/U 5)-administered mice: n = 21, (PCP/Veh)-administered mice: n = 18, (PCP/U 5)-administered mice: n = 18, (PCP/U 5/DPAT 0.05)-administered mice: n = 19]. One-way ANOVA: $F_{(4, 92)} = 20.89$, $p < 0.01$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs corresponding (Sal/Veh)-administered mice, $^{\#\#}p < 0.01$ vs corresponding (PCP/Veh)-administered mice, $^{\$\$}p < 0.01$ vs (PCP/BNS 1/Veh)- or (PCP/U 5/Veh)-administered mice (Tukey's test). Sal: saline, Veh: vehicle, PCP: phencyclidine, BNS: blonanserin, DPAT: 7-OH-DPAT, U: U99194.

show social deficit. This is consistent with previous reports (Wang et al., 2008). It is unlikely that the deficit in these mice is due to changes in the spontaneous activity levels relative to the controls as PCP-administered mice did not show reduced exploratory activity or motor function in the habituation period. Therefore, it is likely that the performance impairment seen in PCP-administered mice was due to a social deficit as one of the negative symptoms of schizophrenia.

Blonanserin inhibits the serotonin 5-HT_{2A} and dopamine-$D_{2/3}$ receptors (Tenjin et al., 2013). Blonanserin significantly ameliorated the PCP-induced social deficit, as measured by the social interaction test. However, at a dose of 1 mg/kg blonanserin had no effect on spontaneous activity during the test session. It is unlikely that the observed amelioration of social deficit was due to changes in spontaneous activity in PCP-administered mice. In contrast to the results following blonanserin treatment, treatment with olanzapine or haloperidol had no ameliorating effect on the PCP-induced social deficit. Olanzapine inhibits the serotonin 5-HT_{2A} and dopamine-D_2, but not D_3 receptors, and haloperidol strongly inhibits the dopamine-D_2, but not D_3 receptors (Hida et al., 2015). Additionally, blonanserin has a higher affinity for the dopamine-D_3 receptors than olanzapine and haloperidol (Ki = 0.49 nM vs 49 nM and 2 nM, respectively; DeLeon et al., 2004; Tenjin et al., 2013). Qiao et al. (2001) reported that haloperidol even at

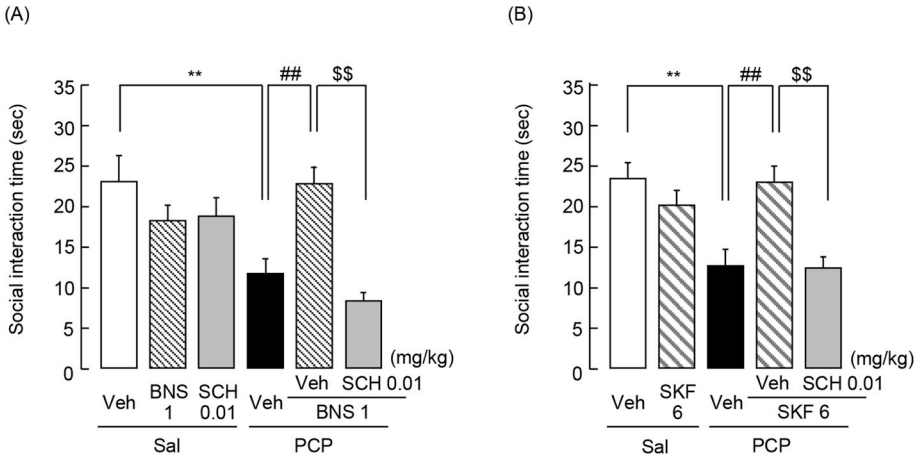

Fig. 3. Involvement of the dopamine-D_1 receptors in the social amelioration effect of blonanserin in PCP-administered mice. Mice were administrated with SCH23390 (0.01 mg/kg, s.c.; A) and SKF38393 (6 mg/kg, i.p.; B) 60 and 30 min, respectively, before the social interaction test session. Social interaction time was measured over a 10-min period. (A) Blonanserin + SCH23390. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 15, (Sal/BNS 1)-administered mice: n = 13, (Sal/SCH 0.01)-administered mice: n = 14, (PCP/Veh)-administered mice: n = 14, (PCP/BNS 1/Veh)-administered mice: n = 13, (PCP/BNS 1/SCH 0.01)-administered mice: n = 12]. One-way ANOVA: $F_{(5, 75)} = 7.79$, $p < 0.01$. (B) SKF38393 + SCH23390. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 8, (Sal/SKF 6)-administered mice: n = 8, (PCP/Veh)-administered mice: n = 7, (PCP/SKF 6/Veh)-administered mice: n = 10, (PCP/SKF 6/SCH 0.01)-administered mice: n = 8]. One-way ANOVA: $F_{(4, 36)} = 9.02$, $p < 0.01$. $^{**}p < 0.01$ vs corresponding (Sal/Veh)-administered mice, $^{\#\#}p < 0.01$ vs corresponding (PCP/Veh)-administered mice, $^{\$\$}p < 0.01$ vs (PCP/BNS 1/Veh)- or (PCP/SKF 6/Veh)-administered mice (Tukey's test). Sal: saline, Veh: vehicle, PCP: phencyclidine, BNS: blonanserin, SCH: SCH23390, SKF: SKF38393.

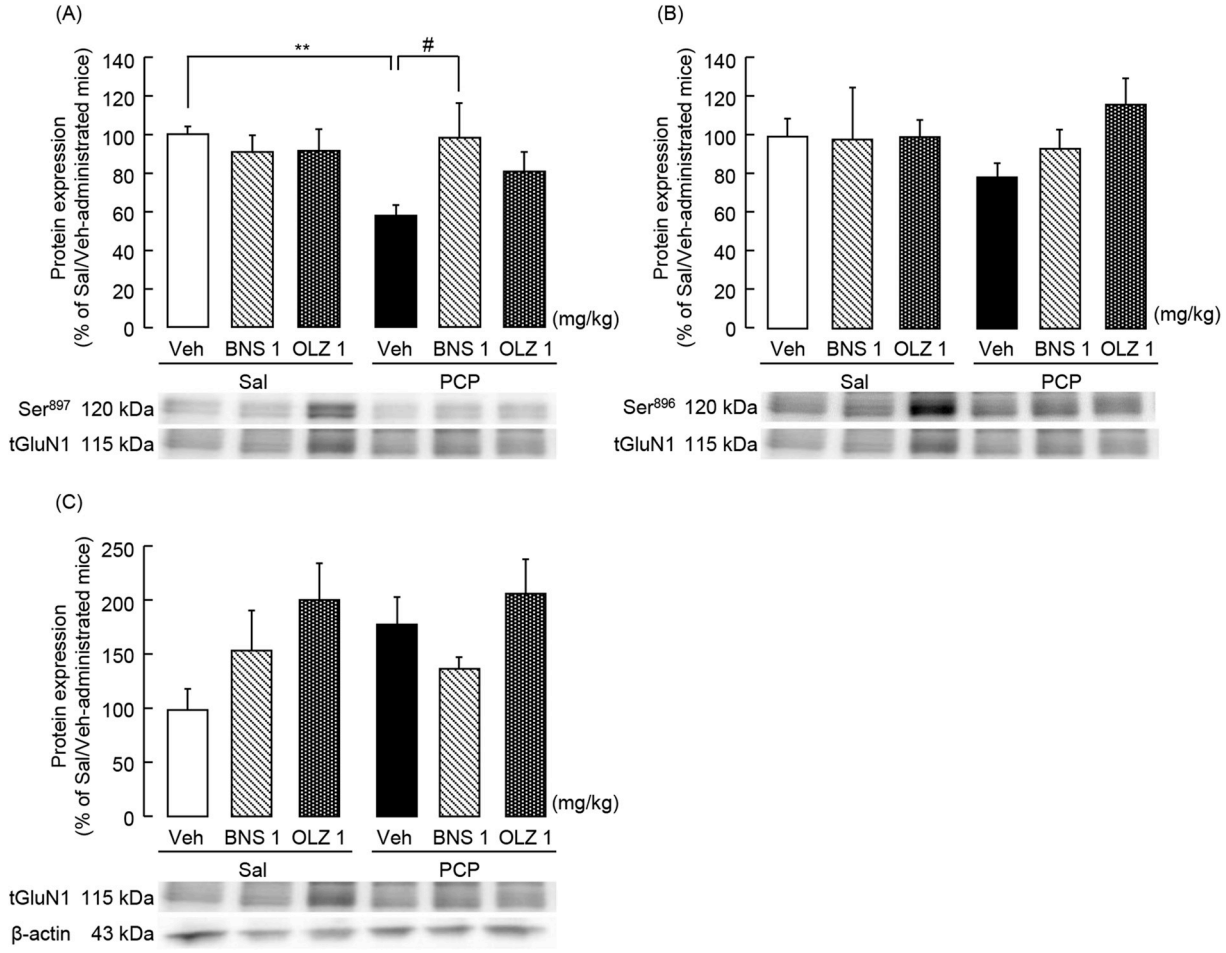

Fig. 4. Effect of blonanserin on Ser^{897}-and Ser^{896}-phosphorylated GluN1 in the PFC of PCP-administered mice. PCP-administered mice were treated with blonanserin (1 mg/kg, p.o.) and olanzapine (1 mg/kg, p.o.) 30 min before the social interaction test session. Immediately after the test session, mice were decapitated. GluN1 (Ser^{896} and Ser^{897}) and total GluN1 expression in the PFC were determined by western blotting analysis. The amount of total GluN1 loaded was normalized using an antibody against β-actin. Values are mean ± S.E.M. [(Sal/Veh)-administered mice: n = 10, (Sal/BNS 1)-administered mice: n = 10, (Sal/OLZ 1)-administered mice: n = 9, (PCP/Veh)-administered mice: n = 10, (PCP/BNS 1)-administered mice: n = 10, (PCP/OLZ 1)-administered mice: n = 11]. One-way ANOVA: (A) Ser^{897}-phosphorylated GluN1/total GluN1; $F_{(5, 54)} = 2.66$, $p < 0.05$. (B) Ser^{896}-phosphorylated GluN1/total GluN1; $F_{(5, 54)} = 0.75$, $p = 0.59$. (C) Total GluN1/β-actin; $F_{(5, 54)} = 2.24$, $p = 0.06$. $^{**}p < 0.01$ vs (Sal/Veh)-administered mice, $^{\#}p < 0.05$ vs (PCP/Veh)-administered mice (Tukey's test). Sal: saline, Veh: vehicle, PCP: phencyclidine, BNS: blonanserin, OLZ: olanzapine, PFC: prefrontal cortex.

3 mg/kg failed to ameliorate the social deficit in PCP-administered mice (Qiao et al., 2001) in consistent with the present finding. Our findings suggest that the dopamine-D_3 receptors, but not the serotonin 5-HT_{2A} and dopamine-D_2 receptors, might be involved in the ameliorating effect of blonanserin on the PCP-induced social deficit. Notably, the ameliorating effect of blonanserin on the PCP-induced social deficit is prevented by the administration of 7-OH-DPAT, but not DOI. Both drugs were administered at doses that did not significantly affect the performance of saline-administered control mice. Furthermore, U99194 also significantly ameliorated the social deficit observed in PCP-administered mice. This effect of U99194 was inhibited by administration of 7-OH-DPAT, while no effect on the performance of saline-administered control mice was observed to 7-OH-DPAT. These effects of 7-OH-DPAT should be interpreted in the context of the known selectivity of this drug for dopamine-D_3 vs D_2 receptors. We suggest that haloperidol, a dopamine-D_2 receptor antagonist, failed to reverse the PCP-induced social deficit, indicating that the dopamine-D_2 receptors are not involved in the amelioration of the PCP-induced impairment. Based on the results from experiments using 7-OH-DPAT and DOI, we demonstrated that the ameliorating effect of the PCP-induced social deficit of blonanserin involves the dopamine-D_3 receptors, but not dopamine-D_2 and serotonin 5-HT_{2A} receptors. However, blonanserin also has a higher affinity for the dopamine-D_2 receptors (Ki = 0.14 nM; Tenjin et al., 2013). Further direct investigation is needed to clarify whether the dopamine-D_2 receptors are involved in the ameliorating effects of blonanserin on the PCP-induced social deficit.

The dopamine-D_3 receptors, on which blonanserin acts, play a dual role as an autoreceptor and a postsynaptic receptor at the dopaminergic synapse (Lévesque et al., 1992; Diaz et al., 1995, 2000). Pharmacological studies suggest that dopamine-D_3 receptors acting as autoreceptors negatively modulate dopamine release (Gross and Drescher, 2012). Antagonism of dopamine-D_3, but not D_2, receptors can also enhance cortical cognitive function by facilitating the release and synthesis of dopamine of the mesocortical dopaminergic system (Gobert et al., 1995; Gross and Drescher, 2012; Watson et al., 2012a, b; Nakajima et al., 2013). Our previous study demonstrated that facilitation of dopamine release through antagonism of the dopamine-D_3 receptors in the mPFC, and the subsequent activation of dopamine-D_1 receptors is required for the improvement of the cognitive impairment seen following blonanserin administration (Hida et al., 2015). Several reports have suggested that the PCP-induced social deficit involves the dopamine-D_1 receptors of the PFC (Wang et al., 2007; Aoyama et al., 2014; Matsumoto et al., 2017). A previous PET study suggested that the dopamine-D_1 and D_2 receptor systems have opposing regulatory

mechanisms in the mediation of pro-social and anti-social behavior in humans (Plavén-Sigray et al., 2014). In the present study, we aimed to ascertain whether the amelioration of the PCP-induced social deficit observed following blonanserin administration was mediated through the dopamine-D_1 receptors, by using SCH23390, a dopamine-D_1 receptor antagonist. The ameliorating effect of blonanserin on the PCP-induced social deficit was completely blocked by pre-administration SCH23390, at doses that did not affect the performance in saline-administered control mice. In addition, SKF38393, a dopamine-D_1 receptor agonist, also improved the PCP-induced social deficit at doses that did not affect the performance in saline-administered control mice. This ameliorating effect of SKF38393 was completely blocked by the pre-administration of SCH23390. Therefore, we confirmed that the ameliorating effect of blonanserin on the PCP-induced social deficit was caused by the activation of the dopamine-D_1 receptors, through dopamine-D_3 receptor antagonism.

Previous studies have shown that cognitive impairments observed in PCP-administered mice are accompanied by the dysfunction of the dopamine-D_1 and/or NMDA receptors in the mPFC (Abekawa et al., 2006). Co-immunoprecipitation studies, using homogenates from the mPFC as well as the hippocampus, have demonstrated that the dopamine-D_1 receptors are close enough to the GluN1 subunit to affect its activity (Kruse et al., 2009). *In vitro* physiological studies, using pyramidal cells from the mPFC of rats, have demonstrated that NMDA receptor functions are facilitated by SKF38393, a dopamine-D_1 receptor agonist, and that this facilitation is dependent on PKA and intracellular calcium (Tseng and O'Donnell, 2004). The protein kinase C (PKC) phosphorylates serine residues 890 and 896, and PKA phosphorylates serine residue 897 of GluN1 subunit (Tingley et al., 1997). In the present study, the abnormally low levels of Ser^{897}-phosphorylated GluN1, which is phosphorylated by PKA, in the PFC of PCP-administered mice were significantly increased by blonanserin treatment. Conversely, olanzapine had no significant effect on the levels of Ser^{897}-phosphorylated GluN1. Furthermore, there was no significant difference in the levels of Ser^{896}-phosphorylated GluN1, which is phosphorylated by PKC rather than PKA, in the PFC in any of the experimental groups. Taken together with previous finding, which *in vivo* dopamine release was facilitated by dopamine-D_3 receptors antagonism of blonanserin (Hida et al., 2015), we speculate that the ameliorating effect of blonanserin on the PCP-induced social impairment is associated with indirect functional stimulation of the dopamine-D_1-PKA-NMDA receptor pathway following augmentation of dopaminergic neurotransmission due to antagonism of dopamine-D_3, but not serotonin 5-HT_{2A} receptors in the mPFC (Supplemental Fig. S3). Since the ameliorating effect of dopamine-D_1 receptor agonist is associated with direct stimulation of the dopamine-D_1-NMDA receptor pathway in the PFC, they as well as dopamine-D_3 receptor antagonists may be useful as a novel treatment strategy. However, further direct investigation exploring the PKA enzyme activity is needed to determine the relationship between the phosphorylated form and enzyme activity of PKA. Further experiments are needed to clarify whether signaling pathways other than the dopamine-D_1 receptor-PKA activation signal are involved in the ameliorating effects of blonanserin on the PCP-induced social deficit.

5. Conclusion

The ameliorating effect of blonanserin on the PCP-induced social deficit is associated with functional stimulation of the dopamine-D_1-NMDA receptor pathway following augmentation of dopaminergic neurotransmission due to inhibition of the dopamine-D_3 receptors in the PFC. These findings also provide *in vivo* evidence that blonanserin antagonism of the dopamine-D_3 receptors may be useful as a novel treatment strategy and that the dopamine-D_3 receptors can be a novel therapeutic target molecule for the social deficits observed in schizophrenia.

Conflicts of interest

Dr. Noda has received research support or speakers' honoraria from Sumitomo Dainippon Pharma, Janssen Pharmaceuticals, Otsuka Pharmaceutical, and Kyorin Pharmaceutical. Dr. Ozaki has received research support or speakers' honoraria from or has served as a consultant to Astellas, Eisai, Eli Lilly, Janssen, Meiji Seika Pharma, Mochida, MSD, Nihon Medi-Physics, Novartis, Ono, Kyowa Hakko Kirin, Otsuka, Pfizer, Takeda, Taisho, Mitsubishi Tanabe, Tsumura, and KAITEKI.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.04.008.

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