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KKHA-761, a potent D_3 receptor antagonist with high 5-HT_{1A} receptor affinity, exhibits antipsychotic properties in animal models of schizophrenia

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Abstract

KKHA-761, 1-{4-[3-(3,4-dimethoxy-phenyl)-isoxazol-5-yl]-butyl}-4-(2-methoxy-phenyl)-piperazine, has a high affinity (K_i=3.85 nM) for human dopamine D_3 receptor with about 70-fold selectivity over the human dopamine D_{2L} receptor (K_i=270 nM). KKHA-761 also showed high affinity for cloned human 5-HT_{1A} receptor (K_i=6.4 nM). KKHA-761 exhibited D_3 and 5-HT_{1A} receptor antagonist activities in vitro, reversing dopamine- or 5-HT-mediated stimulation of [^{35}S]GTPrS binding. The in vivo pharmacological profile of KKHA-761 was compared with both typical and atypical antipsychotics including clozapine and haloperidol. Apomorphine-induced dopaminergic behavior, cage climbing, in mice was potently blocked by a single administration (i.p.) of KKHA-761 (ID_{50}=4.06 mg/kg) or clozapine (ID_{50}=4.0 mg/kg). Cocaine- or MK-801-induced hyperactivity in animals was markedly inhibited by KKHA-761 or clozapine. In addition, KKHA-761 significantly reversed the disruption of prepulse inhibition (PPI) produced by apomorphine in mice, indicating the antidopaminergic or antipsychotic activity of KKHA-761 in mice. However, KKHA-761 was inactive in the forced swimming behavioral despair model in mice, suggesting lack of antidepressant properties. KKHA-761 attenuated the hypothermia induced by a selective dopamine D_3 agonist, 7-OH-DPAT, in mice, whereas clozapine enhanced it. Moderate doses of both KKHA-761 and clozapine did not increase serum prolactin levels in rats. Lower doses of, however, haloperidol significantly increased prolactin secretion. KKHA-761 did not induce cataleptic response up to 20 mg/kg, but significant catalepsy was shown at lower doses of clozapine and haloperidol. Furthermore, KKHA-761 showed a low incidence of rotarod ataxia (TD_{50}=34.4 mg/kg, i.p.) in mice. The present results, therefore, suggest that KKHA-761 is a potent antipsychotic agent with combined dopamine D_3 and serotonin 5-HT_{1A} receptors modulation activity, which may further enhance its therapeutic potential for anxiety, psychotic depression, and other related disorders.

Keywords: KKHA-761; Dopamine D_3 antagonist; Serotonin 5-HT_{1A} receptor; Antipsychotics; Schizophrenia

1. Introduction

Schizophrenia is characterized by a diversity of symptoms of positive, negative, and cognitive-attentional. It has been reported that dopaminergic systems play an important role in regulation of neuropsychiatric disorders such as schizophrenia (Seeman and Van Tol, 1994; Carlsson, 1988), and conventionally, schizophrenia has been treated with dopaminergic D_2 receptor blockers such as haloperidol. However, a significant population of patients is still refractory to treatment. Furthermore, prominent dopaminergic D_2 antagonists have little effects on negative and cognitive symptoms of psychosis, and elicit extrapyramidal side effects in humans.

In the early 1990s, the cloning of the five identified subtypes of dopamine receptors, especially D_3 and D_4, allowed for new insights into the wider diversity of the actions of dopamine. And the dopamine D_3 receptor has been identified as a novel target for the improved drug treatment of psychosis like schizophrenia (Sokoloff et al., 1990). Following the proposal that D_3 receptors may be of particular significance for neuropsychiatric diseases, several compounds have recently been developed with significantly greater D_3 receptor selectivity and reduced liability for extrapyramidal side effects (Millan et al., 2000; Reavill et al., 2000; Carr et al., 2002). However, the pharmacological approaches to understand

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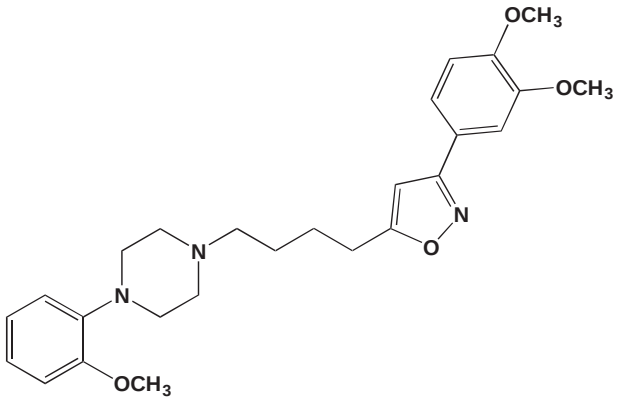

Fig. 1. Chemical structure of KKHA-761.

dopamine D_3 receptor functions have been limited because of a lack of specific ligands for the D_3 receptor. For the characterization of, therefore, the physiological and therapeutic significances of dopamine D_3 receptor, chemically diverse, potent and selective dopamine D_3 receptor antagonists should be still developed.

Meanwhile, there has been increasing interest in the role of the 5-HT system in the modulation of dopamine neurotransmission, and the dopaminergic atypical antipsychotic, clozapine, has also been shown to be a modulator of 5-HT_{1A}, 5-HT_{2A} or 5-HT_6 receptor (Meltzer, 1995). Agonistic properties of clozapine at 5-HT_{1A} autoreceptors may contribute to its effects on clinical improvements (Millan, 2000). In addition, serotonin 5-HT_{1A} receptor agonists attenuate induction of dopamine release in the nucleus accumbens by amphetamine (Ichikawa et al., 1995). Although there were no consistent pattern of data in neurochemical and behavioral studies, modulations of dopaminergic transmission by serotonergic 5-HT_{1A} agonists may enhance antipsychotic efficacy in resistant patients. The serotonergic 5-HT_1 and 5-HT_2 receptors also have been regarded to be involved in the etiology of anxiety and depression. Thus, it has been postulated that the antipsychotics with moderate affinity to serotonin 5-HT_1 or 5-HT_2 receptor could potentially alleviate anxiogenic or depressant effect as well as negative symptoms associated with schizophrenia. Currently, the development of antipsychotic drugs which modify both dopamine and serotonin receptors is one of the potential approaches to the treatment of schizophrenia.

The present study was designed to evaluate the antipsychotic properties of newly synthesized dopamine D_3 receptor antagonist with high affinity for 5-HT_{1A} receptor, KKHA-761, 1-{4-[3-(3,4-dimethoxy-phenyl)-isoxazol-5-yl]-butyl}-4-(2-methoxy-phenyl)-piperazine (Fig. 1).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 210–260 g and ICR male mice (21–25 g) were obtained from Orient (Orient Corporation, Seoul, Korea). They were housed in a humidity (60±5%) and temperature (21±1 °C)-controlled animal facility on a 12:12 h light/dark cycle and were given a solid diet and tap water ad libitum. All experimental procedures were performed during the light cycle, and all experimental protocols were consistent with the guidelines issued by the National Institutes of Health (USA) and Institutional Animal Care and Use Committee of Korea Research Institute of Chemical Technology.

2.2. Drugs

Haloperidol, clozapine, apomorphine hydrochloride, (+)-MK801 hydrogen maleate, *R*(+)-7-OH-DPAT hydrobromide and imipramine were from Sigma (St. Louis, USA), and cocaine hydrochloride was purchased from Macfarlan Smith Ltd. (Edinburgh, UK). Radioligands, [^{3}H]Spiperone, [^{3}H]YM-09151-2, [^{3}H]Ketanserin, [^{3}H]LSD, and [^{35}S]GTPrS were purchased from PerkinElmer (PerkinElmer Life and Analytical Sciences, Boston, USA), whereas [^{3}H]8-OH-DPAT and [^{3}H]Mersulergin were obtained from Amersham Biosciences (Buckinghamshire, UK). Cloned human D_{2L}, $D_{4.2}$, 5-HT_6, 5-HT_7, and rat D_3 receptors were obtained from PerkinElmer, and human serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors were purchased from Euroscreen (Brussels, Belgium). Cloned human D_3 receptor membranes were constructed at our laboratory of Korea Research Institute of Chemical Technology.

2.3. Cloned cell lines expressing human dopamine D3 receptor

Human dopamine D_3 receptor protein was expressed in insect cell. Briefly, human D_3 cDNA was cloned from human brain cDNA library (Clontech, Palo Alto, USA) by PCR amplification using 5′-GCTATGGCATCTCTGAGTCA-3′ for forward and 5′-GGAGACACTGCTCTGTCTTT-3′ for reverse. Amplified cDNA fragments were introduced into pGEMT easy vector (Promega, Medison, USA) and then DNA sequencing was performed to confirm receptor DNA sequence. Dopamine D_3 clone was subcloned into insect cell expression vector BacPAK8 (Clontech). pBacPAK8/D_3 was transfected into insect *Sf*21 cells (Clontech) and protein expression of D_3 receptor was confirmed by SDS PAGE and receptor binding assay. Cell lysis was performed by sonication for 2 min at 4 °C and cell debris was discarded by centrifugation for 10 min at 3000 $\times g$. Membrane fraction was purified partially from supernatant above by centrifugation for 1 h at 100,000 $\times g$.

2.4. Radioligand binding assays

2.4.1. [^{3}H]Spiperone binding to dopamine D2L and D3 receptors

Competition binding assays at dopamine D_{2L} and D_3 receptors were performed using 0.5 and 0.8 nM of [^{3}H]Spiperone, respectively, by the protocols provided by the supplier of Sf-9 membranes (PerkinElmer Life and Analytical Sciences) with minor modifications. Briefly, receptor membranes were incubated at 27 °C for 60 min in a final volume of 0.25 ml

reaction mixture containing [^{3}H]Spiperone and various concentrations of the drug in 50 mM Tris–HCl (pH 7.4) buffer containing 10 mM $MgCl_2$, 1 mM EDTA and 120 mM NaCl. Then, the incubations were terminated by rapid filtration using an Innotech cell harvester (Innotech Biosystems, Switzerland) through Whatman GF/C glass fiber filter presoaked in 0.3% polyethylenimine. The filter was covered with MeltiLex, sealed in a sample bag followed by drying in the microwave oven, and counted by MicroBeta Plus (Wallac, Finland). Nonspecific binding was determined in the presence of 10 μM haloperidol. Competition binding studies were carried out with 7–8 varied concentrations of the test compounds run in duplicate tubes, and isotherms from three assays were calculated by computerized nonlinear regression analysis (GraphPad Prism Program, San Diego, USA) to yield inhibition constant (K_i) values.

2.4.2. [^{3}H]YM-09151-2 binding to dopamine D4.2 receptor

In [^{3}H] YM-09151-2 receptor binding assays, cloned dopamine $D_{4.2}$ receptor membranes prepared from Sf9 cells and 0.1 nM of [^{3}H] YM-09151-2 were incubated in the buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM KCl and 1.5 mM $CaCl_2$. Nonspecific binding was defined with clozapine (10 μM) for $D_{4.2}$ receptors. After incubation for 60 min at 27 °C, assay was terminated by rapid filtration through Whatman GF/C glass fiber filter presoaked in 0.3% polyethylenimine.

2.4.3. [^{3}H]8-OH-DPAT binding to serotonin 5-HT1A receptor

Membranes from stable CHO-K1 cell line expressing the human recombinant 5-HT_{1A} serotonin receptor were used. For the binding assay, aliquots of receptor membranes, 0.5 nM [^{3}H]8-OH-DPAT and appropriate concentrations of test compounds were added to 0.25 ml of 50 mM Tris–HCl (pH 7.4) buffer containing 1 mM EDTA and 2.5 mM $MgCl_2$. Nonspecific binding was determined using 0.5 μM methiothepin. Incubations were carried out for 30 min at 37 °C, and these were terminated by rapid filtration using an Inotech cell harvester through Whatman GF/C glass fiber filter presoaked in 0.3% polyethylenimine. The filter was covered with MeltiLex, sealed in a sample bag followed by drying in the microwave oven, and counted by MicroBeta Plus.

2.4.4. [^{3}H]Ketanserin binding to serotonin 5-HT2A receptor

For serotonin 5-HT_{2A} binding, an aliquot of frozen membrane from CHO-K1 cell line expressing the human recombinant 5-HT_{2A} receptor and [^{3}H]Ketanserin (1 nM) were used in the presence of mianserin (0.5 μM) as nonspecific. The reaction mixture was incubated for 15 min at 37 °C using 50 mM Tris–HCl (pH 7.4) buffer, and harvested through Whatman GF/C glass fiber filter presoaked in 0.05% Brij.

2.4.5. [^{3}H]Mesulergine binding to serotonin 5-HT2C receptor

Frozen membranes from stable CHO-K1 cell line expressing the human recombinant 5-HT_{2C} receptor were used. For the binding assay, [^{3}H]Mesulergine (1 nM), receptor membrane and test compounds were added into 50 mM Tris–HCl (pH 7.7) buffer containing 0.1% ascorbic acid and 10 μM pargyline. Nonspecific binding was determined using 0.5 μM mianserin. The incubations were performed for 30 min at 37 °C, and these were terminated by rapid filtration through Whatman GF/C glass fiber filter presoaked in 1% BSA.

2.4.6. [^{3}H]LSD binding to serotonin 5-HT6 and 5-HT7 receptor

For receptor binding assays, human 5-HT_6 and 5-HT_7 serotonin receptor expressed in HeLa or CHO cells, respectively, were used. For 5-HT_6 receptor binding, frozen membrane, 1.8 nM [^{3}H]LSD and appropriate concentrations of test compounds were added to 0.25 ml of assay buffer. Incubations were carried out for 60 min at 37 °C, and these were terminated by rapid filtration through Whatman GF/C glass fiber filter presoaked in 0.3% polyethylenimine.

For 5-HT_7 receptor binding assay, cell membrane, 3 nM [^{3}H]LSD and appropriate concentrations of test compounds were added to 0.25 ml of assay buffer. The mixture was incubated for 90 min at 27 °C, and the reaction was terminated by rapid filtration through Whatman GF/C glass fiber filter presoaked in 0.3% polyethylenimine. Methiothepin and 50 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$ and 0.5 mM EDTA, were used as the nonspecific ligand or assay buffer, respectively, for these serotonergic receptor binding assays.

2.4.7. Radioligand binding to other receptors, ion channels or enzymes

Radioligand binding assays also were performed on 27 receptors, ion channels, and enzyme binding sites by MDS Pharma Services (Bothell, WA, USA).

2.5. In vitro function study: [^{35}S]GTPrS binding

[^{35}S]GTPrS bindings at D_3 and 5-HT_{1A} receptors were performed by MDS Pharma Services with the modified methods of Newman-Tancredi et al. (1999) and Adlersberg et al. (2000), respectively. Membranes from stable CHO-K1 cell line expressing the human D_3 or 5-HT_{1A} receptor were used. For the binding assay, aliquots of receptor membrane, [^{35}S]GTPrS (1250 Ci/mmol; PerkinElmer) and appropriate concentrations of KKHA-761 were added to 20 mM HEPES (pH 7.4) buffer containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 3 μM GDP. Nonspecific binding was determined using 10 μM GTPrS. Incubations were performed for 30 min at 30 °C. For antagonist studies, inhibitory actions of KKHA-761 on dopamine (1 μM)- and 5-HT (10 μM)-stimulated [^{35}S]GTPrS binding at D_3 or 5-HT_{1A} receptor, respectively, were determined.

2.6. Apomorphine-induced climbing behavior in mice

Mice were injected with apomorphine (1 mg/kg, s.c.), and were put into cylindrical cages with the floor and wall

consisting of metal bars and covered with a lid. After a 5-min period of exploratory activity, climbing behavior was scored by an observer who was blind to the drug treatment at 10, 20 and 30 min after apomorphine administration (Kim and Park, 1995; Kim et al., 1996). The scores of this behavior were evaluated as follows: normal behavior (0 point), increased activity and sniffing (1 point), occasional clinging to sides of cage with forepaws (2 points), intermittent clinging to sides of top of cage with all four paws (3 points) and uninterrupted climbing with all four paws (4 points). KKHA-761 (0.5, 1, 2.5, 5, 10, 20, 30 or 50 mg/kg), clozapine (1, 2.5, 5 or 10 mg/kg) or haloperidol (0.05, 0.1 or 0.2 mg/kg) was suspended in 3%-Tween 80 solution, and administered intraperitoneally or orally to mice 30 or 60 min before the injection of apomorphine, respectively. Apomorphine was dissolved in 0.9% physiological saline containing 0.1% ascorbic acid. The median inhibitory dose (ID_{50}) with 95%-confidence limits was calculated to estimate drug potency.

2.7. Cocaine and MK801-induced locomotor activities

Cocaine-induced hyperlocomotion in mice was determined using Activity Analyzer (AM1052 Activity Monitor, Benwick Electronics, Benwick, UK). On the testing day, mice were placed in transparent polycarbonate cages located in activity chambers and were allowed to habituate for 30 min. For cocaine-induced locomotor activity, vehicle, KKHA-761 (1, 2.5 or 5 mg/kg) or clozapine (1 or 5 mg/kg) was pretreated (s.c.) 30 min prior to the injection of cocaine (20 mg/kg, i.p.) or saline (0.2 ml/20 g, i.p.), and activity counts were recorded every 10 min for 1 h immediately after injection of cocaine or saline.

For MK801-induced locomotor activity, rats were pretreated (i.p.) with KKHA-761 (2.5 or 5 mg/kg) or vehicle 30 min before the injection of MK801 (0.2 mg/kg, s.c.) or saline (0.2 ml/200 g, s.c.), and the distance traveled was recorded every 10 min for 1 h immediately after injection of MK801 or saline using Tru Scan Activity (Coulbourn, Allentown, USA).

KKHA-761 and clozapine were suspended in 3%-Tween 80 solution, whereas MK801 and cocaine were dissolved in 0.9% physiological saline.

2.8. Prepulse inhibition (PPI)

Startle response was measured using SR-LAB startle chamber (San Diego Instruments, San Diego, USA). The animal enclosure was housed in a ventilated and sound-attenuated startle chamber with 60 dB ambient noise level, and consisted of a Plexiglas cylinder 40 mm in diameter on a platform, connected to a piezoelectric accelerometer which detects and transduces motion within the cylinder. Acoustic noise bursts were presented through a loudspeaker mounted 24 cm above the animal.

Behavioral testing was performed between 10 a.m. and 5 p.m., during the light phase by a modified method of Mansbach et al's (1998). Each startle session began with a 5-min acclimatizaton period in the chamber to 68 dB background noise. The test session consisting of the following four different trial types was carried out for all experiments: a 40 ms broadband 120 dB burst (P; pulse alone trial), P preceded 100 ms earlier by a 20 ms noise burst 10 dB above background (pP; prepulse + pulse trial), a 40 ms broadband 78 dB burst (prepulse alone trial), and a no stimulus trial (background). Eight trials of each type were presented in a pseudorandom order (total 32 trials) with an average interval of 15 s separating each trial. An extra 5 pulse-alone trials were presented at the beginning and end of each test session, but were not used in the calculation of PPI values. PPI was defined as the percent reduction in startle amplitude in the presence of prepulse compared to the amplitude in the absence of the prepulse using the formula PPI (%) = [100 − (100 × startle amplitude on pP trial/startle amplitude on P trial)]. The mice were administered (i.p.) with either vehicle or KKHA-761 (5 and 10 mg/kg) 30 min before the injection of apomorphine (3 mg/kg, s.c.), and were placed in the startle chamber 30 min after the apomorphine injection for testing.

2.9. Forced swimming in mice

The forced swimming test was performed according to the methods described by Porsolt et al. (1978). Each mouse was placed in a 25-cm glass cylinder (10 cm diameter) containing 15 cm of water maintained at 23 ± 1 °C, and was forced to swim for 10 min. Twenty-four hours later, the mouse was replaced into the cylinder and the total duration of immobility was recorded during the last 5 min of the 6-min testing period. Mice are judged immobile when they float in an upright position and make only small movements to keep their head above water. KKHA-761 (1, 5, 10 and 20 mg/kg) and clozapine (1, 2.5 and 5 mg/kg) were suspended in 3%-Tween 80 solution, and administered (i.p.) 30 min before the testing.

2.10. Hypothermia induced by 7-OH-DPAT in mice

Body temperature (baseline) of mouse was determined 30–60 min before the administration of the test compound, and the mice were injected (i.p.) with vehicle, KKHA-761 (2.5 or 5 mg/kg) or clozapine (5 or 10 mg/kg), followed 30 min later by an injection of 7-OH-DPAT (0.2 mg/kg, s.c.) dissolved in saline. After 30 min, rectal temperature was again determined by use of digital telethermometer (Thermalert TH-5, Physitemp Instruments Inc., Clifton, USA) and the difference in temperature to baseline value was calculated.

2.11. Prolactin radioimmunoassay in rats

KKHA-761 (1, 3, 10 or 50 mg/kg), clozapine (2.5 or 10 mg/kg), haloperidol (0.3, 1 or 3 mg/kg) or vehicle was administered (i.p.) into the rats, and after 2 h the animals were decapitated and the blood was collected into glass vials containing heparin. Blood samples were centrifuged immediately to remove cells and the plasma was kept below −15 °C

Table 1
Binding affinity of KKHA-761 for the cloned dopamine and serotonin receptors

Receptor	[^{3}H]Ligand	Cells	K_i (nM)
hD_{2L}	Spiperone	SF9	270.6±66.5
hD_3	Spiperone	SF21	3.85±1.01
rD_3	Spiperone	SF9	5.14±1.25
$hD_{4.2}$	YM-09151-2	SF9	154.2±37.7
h5-HT_{1A}	8-OH-DPAT	CHO-K1	6.4±1.06
h5-HT_{2A}	Ketanserin	CHO-K1	72.7±12.4
h5-HT_{2C}	Mesulergine	CHO-K1	95.9±21.3
h5-HT_6	LSD	HeLa	501.9±132.3
h5-HT_7	LSD	CHO	76.3±14.5

Values are means±S.E.M. of three separate competition experiments.
h=cloned human receptor. r=cloned rat receptor.

prior to analysis. Plasma concentrations of prolactin (PRL) were measured using the Biotrak™ rat PRL [^{125}I] assay system from Amersham Biosciences.

2.12. Catalepsy in mice

The animals were placed individually in clear acrylic cages and allowed a minimum 30 min to acclimatize to the new environment. Catalepsy was assessed by positioning mice with their hindpaws on the floor and their forelimbs rested on an elevated bar. The time that the paws remained on the bar was determined up to a maximum of 30 s. The mean value of three tests separated by intervals of 1 min was recorded (Millan et al., 1998a). Vehicle, KKHA-761 (5, 10, 20 or 30 mg/kg), clozapine (2.5, 5, 10 or 30 mg/kg) or haloperidol (0.1, 0.25, 0.5, 1 or 2 mg/kg) was injected (i.p.) into the mice, and 30 or 60 min later, the catalepsy was scored.

2.13. Rotarod and lethality in mice

The mouse was placed on a 1 in. diameter knurled plastic rod rotating at 6 rpm (Ugo-Basile, Milano, Italy), and the rotarod deficit (%) was obtained by counting the number of animals fallen from the rotating rod within 1 min (Dunham et al., 1957). The median neurotoxic dose (TD_{50}) was determined as the dose at which 50% of animals showed rotarod deficit. The KKHA-761, clozapine or vehicle was administered (i.p.) 30 min before the testing.

Lethality was scored 48 h after the single administration of the compound, and the median lethal dose (LD_{50}) was determined as the dose which causes the death of 50% of a group of test animals. KKHA-761 or clozapine was suspended in 3%-Tween 80 solution, and administered intraperitoneally.

2.14. Statistical analysis

Statistical significance of the results was evaluated by one-way analysis of variance (ANOVA) with Dunnett's post-hoc tests for comparing control to treatment, except for the tests of climbing and catalepsy behaviors, which were analyzed by the Kruskal–Wallis ANOVA on ranks, followed by the nonparametric Dunnett's test. Differences were considered significant at $P<0.05$. Statistical analyses were conducted using SigmaStat software (SigmaStat, Jandel Co., San Rafael, CA). The data were expressed as means±S.E.M.

3. Results

3.1. Radioligand binding studies

KKHA-761 had high affinity for the human D_3 (hD_3) receptor with K_i value of 3.85±1.01 nM, and had much lower affinity for the hD_2 receptor (K_i=270.6±66.5 nM). KKHA-761, therefore, showed about 70-fold selectivity for the D_3 receptor over the D_2 receptor. KKHA-761 also bound potently to the rat D_3 receptor (K_i=5.14±1.25 nM), whereas the $hD_{4.2}$ receptor was moderately inhibited showing only 154.2±37.7 nM of K_i value (Table 1).

Like many other antipsychotics such as clozapine or ziprasidone, KKHA-761 had moderate affinity for serotonin receptors. Among these receptors, KKHA-761 bound with high affinity to the human serotonin 5-HT_{1A} receptor (K_i=6.4±1.06 nM). In contrast, KKHA-761 had relatively weak affinities for human 5-HT_{2A} (72.7±12.4 nM), 5-HT_{2C} (95.9±21.3 nM), 5-HT_6 (501.9±132.3 nM), and 5-HT_7 (76.3±14.5 nM) receptors.

KKHA-761 also was tested at 27 receptors, ion channels, and enzyme binding sites (MDS Pharma Services; MDSPS PT # 1064545). KKHA-761 (10 μM) produced less than 20% inhibition of specific binding at adenosine A_1 and A_{2A}, $GABA_A$ (agonist site) and $GABA_A$ (benzodiazepine), NMDA receptor phencyclidine site, Muscarinic M_2 and M_3, opiate μ, phorbol ester, potassium channel [K_{ATP}], prostanoid EP_4 and rolipram. KKHA-761 showed 20~60% inhibition of [^{35}S]GTPrS binding at calcium channel L-type, nicotinic acetylcholine and norepi-

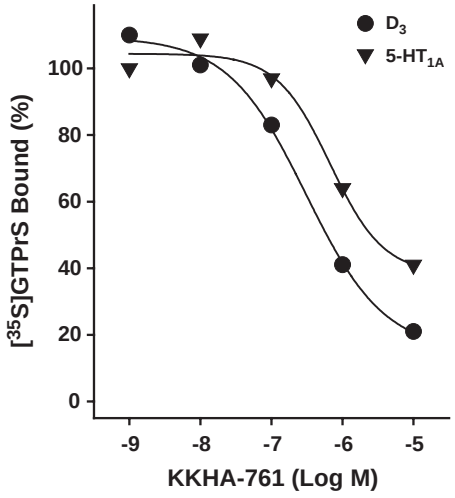

Fig. 2. Antagonism of agonist-stimulated [^{35}S]GTPrS binding in CHO-K1 cells transfected with the human D_3 or 5-HT_{1A} receptor by KKHA-761. Agonist-stimulated [^{35}S]GTPrS binding was determined in the presence of dopamine (1 μM) or 5-HT (10 μM) for D_3 or 5-HT_{1A} receptor, respectively. The data are expressed as percent of maximal [^{35}S]GTPrS bound by each agonist in the absence of KKHA-761.

Table 2
Inhibition by KKHA-761, clozapine or haloperidol of apomorphine-induced climbing behavior in mice

Treatment	ID_{50} (mg/kg)	
	i.p.	p.o.
KKHA-761	4.06 (3.0~5.4)[a]	16.9 (15.5~18.6)
Clozapine	4.0 (2.3~6.9)	n.d.
Haloperidol	0.07 (0.04~0.13)	n.d.

Apomorphine (1 mg/kg, s.c.) was injected 30 (i.p.) or 60 (p.o.) min after administration of the drugs, and climbing behavior was measured 10, 20 and 30 min after apomorphine injection using the 4-point rating scale. The median inhibitory dose (ID_{50}) with 95% confidence limits was calculated by nonlinear regression analysis. [a]95% confidence limits. n.d.; not determined.

nephrine transporter. KKHA-761 (10 μM) also showed more than 60% inhibition of specific binding at the remaining sites such as α_{1A}, α_{1B}, β_1, and β_2 adrenoceptors, histamine H_1, sigma σ_1 and σ_2, sodium channel site 2.

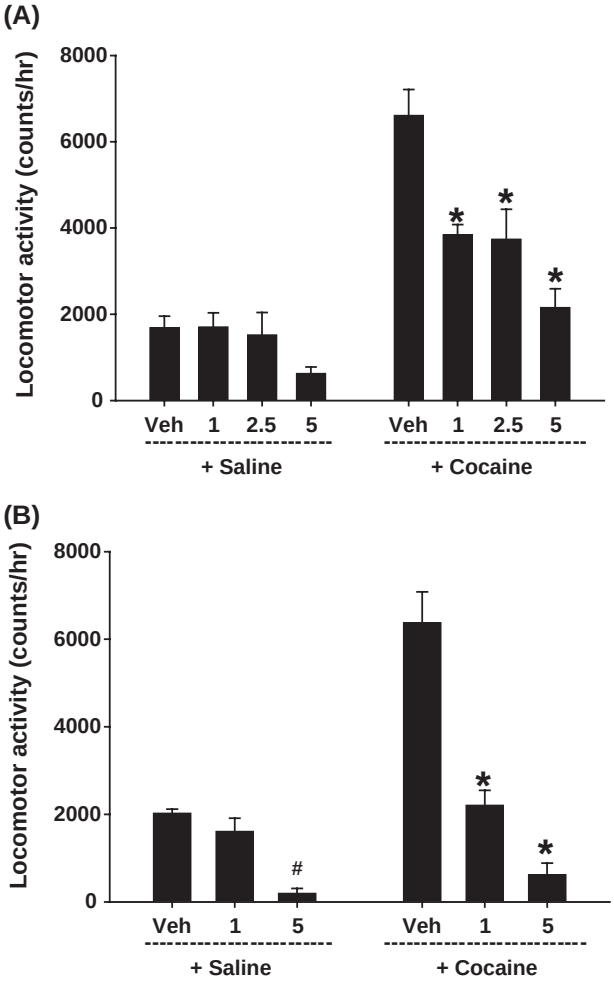

Fig. 3. Effects of KKHA-761 and clozapine on spontaneous and cocaine-induced locomotor activity in mice. (A) KKHA-761 (1, 2.5 and 5 mg/kg) or (B) Clozapine (1 and 5 mg/kg) was injected (s.c.) 30 min before the injection of cocaine (20 mg/kg, i.p.) or saline (0.2 ml/20 g, i.p.), and the locomotor activity was measured for 1 h immediately after injection of cocaine or saline. Each value is a mean±S.E.M. $^{*}P<0.05$, $^{\#}P<0.05$, compared with corresponding vehicle (Veh) group, respectively (Dunnett's test).

3.2. Effects on agonist-stimulated [^{35}S]GTPrS binding

Dopamine produced concentration-dependent increase in [^{35}S]GTPrS binding in membranes prepared from CHO-K1 cells expressing the human D_3 receptor (data not shown), and this increase was concentration-dependently suppressed by KKHA-761 with an IC_{50} value of 0.80 μM (Fig. 2). A similar pattern of data was acquired for [^{35}S]GTPrS binding at serotonin 5-HT_{1A} receptor, and its IC_{50} value appeared to 4.3 μM (MDS Pharma Services; MDSPS PT# 1064545).

3.3. Effects on apomorphine-induced climbing behaviors

Apomorphine (1 mg/kg, s.c.)-induced climbing behavior was potently blocked by KKHA-761 (2.5, 5, 10 and 20 mg/kg, i.p.) in mice [$H(6)=32.18$, $P<0.001$] without any rotarod ataxia, and thus its median inhibitory dose (ID_{50}) was 4.06 mg/kg (95% confidence limit=3.0–5.4 mg/kg). Orally administered KKHA-761 also inhibited the apomorphine-induced climbing in mice (ID_{50}=16.9 mg/kg; 95% confidence limit=15.5–18.6 mg/kg). In addition, apomorphine-induced climbing behavior was significantly suppressed by clozapine or haloperidol, showing 4.0 and 0.07 mg/kg of ID_{50} values, respectively (Table 2).

3.4. Effects on cocaine and MK801-induced locomotor activity in mice or rats

KKHA-761 (1, 2.5 and 5 mg/kg, s.c.) alone manifested no overall treatment effect in spontaneous locomotor activity in mice [$F(3,16)=2.239$, $P=0.123$]. Cocaine (20 mg/kg, i.p.)-induced hyperactivity, however, was significantly [$F(3,20)=12.8$, $P<0.001$] inhibited by KKHA-761 in mice (Fig. 3A). In addition, clozapine (1 and 5 mg/kg, s.c.) also significantly inhibited the cocaine-induced hyperactivity [$F(2,15)=39.1$,

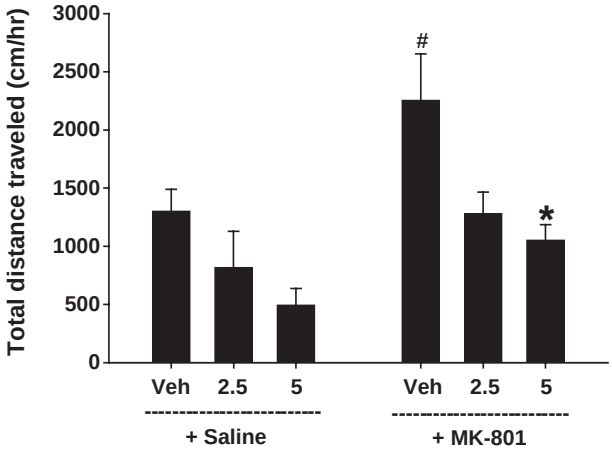

Fig. 4. Effect of KKHA-761 on MK801-induced hyperlocomotion in rats. KKHA-761 (2.5 and 5 mg/kg) or vehicle (Veh) was administered (i.p.) into rats 30 min before the injection of MK801 (0.2 mg/kg, s.c.) or saline, and the distance traveled was measured for 1 h immediately after the injection of MK801 or saline. Values are means±S.E.M. $^{*}P<0.05$, $^{\#}P<0.05$, when compared to the MK801-treated or saline-treated control group, respectively (Dunnett's test).

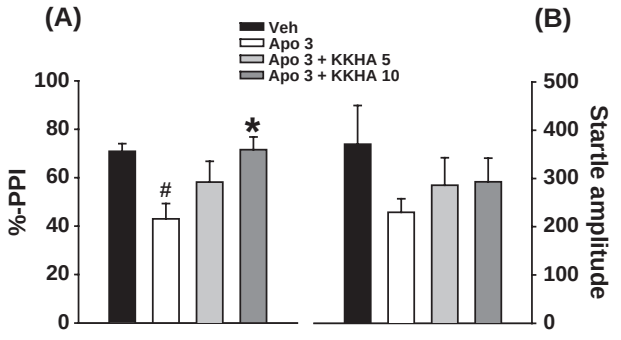

Fig. 5. (A) Effects of KKHA-761 on apomorphine-induced disruption of prepulse inhibition in mice. (B) Effects of KKHA-761 on startle amplitude. KKHA-761 (5 and 10 mg/kg) or vehicle was pretreated (i.p.) 30 min before the injection of apomorphine (3 mg/kg, s.c.). Values are means ± S.E.M. $^{*}P<0.05$, $^{\#}P<0.05$, when compared to the apomorphine (Apo)-treated or vehicle (Veh)-treated control group, respectively (Dunnett's test).

$P<0.001$], and 5 mg/kg of clozapine alone inhibited ($P<0.005$) the spontaneous locomotor activity in mice (Fig. 3B).

In the MK801-induced locomotor activity experiment, MK801 (0.2 mg/kg, s.c.) produced significant enhancement in locomotor activity in rats, when compared to saline control group ($P<0.05$). Spontaneous locomotor activity in rats was not changed significantly by treatment with KKHA-761 (2.5 and 5 mg/kg, i.p.) alone [$F(2,14)=3.628$, $P=0.054$]. MK801-induced locomotor stimulation, however, was significantly antagonized by 5 mg/kg of KKHA-761 ($P<0.05$) in rats (Fig. 4).

3.5. Effects on apomorphine-induced disruption of prepulse inhibition (PPI)

KKHA-761 (5 and 10 mg/kg, i.p.) alone had no significant effect on PPI when compared to vehicle control in mice [$F(2,27)=0.755$, $P=0.479$]. However, the disruption of PPI by apomorphine (3 mg/kg, s.c.) was reversed significantly by pretreatment with KKHA-761 [$F(2,24)=4.221$, $P=0.027$], and post hoc comparisons indicated that 10 mg/kg of KKHA-761 significantly ($P<0.05$) blocked the apomorphine-induced disruption of PPI (Fig. 5A). There were no significant differences in mean startle amplitude of KKHA-761 (5 or 10 mg/kg, i.p.) administered 30 min before apomorphine when compared with that of apomorphine control group [$F(2,24)=0.612$, $P=0.551$] (Fig. 5B). In addition, KKHA-761 (5 or 10 mg/kg, i.p.) alone had no significant effects on the mean startle response compared to vehicle control [$F(2,27)=1.024$, $P=0.373$]. In the preliminary study, the blockade of PPI by apomorphine was significant for the 3 mg/kg (s.c.) dose when compared with vehicle control ($P<0.001$). Thus, 3 mg/kg of apomorphine was selected for this PPI test.

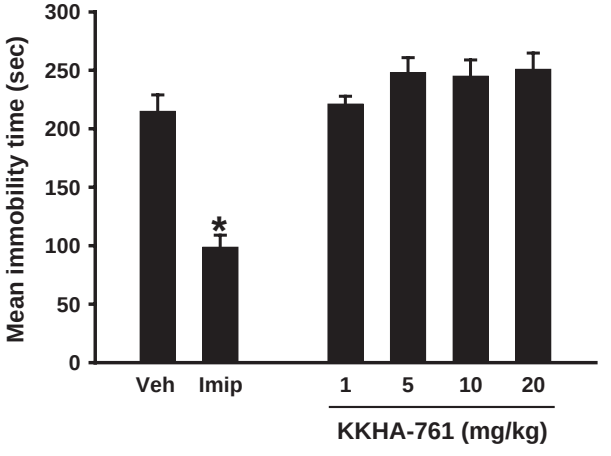

Fig. 6. Effects of KKHA-761 and imipramine on immobility in forced swimming test in mice. KKHA-761 (1, 5, 10 and 20 mg/kg) and imipramine (Imip, 25 mg/kg) were injected (i.p.) 30 min before the testing, and the total duration of immobility was recorded during the last 5 min of the 6-min testing period. Values are means ± S.E.M. $^{*}P<0.05$, when compared to the vehicle (Veh)-treated control group (Student t test).

3.6. Effects on forced swimming in mice

A single administration of KKHA-761 (1, 5, 10 and 20 mg/kg, i.p.) did not affect immobility duration [$F(4,55)=1.676$,

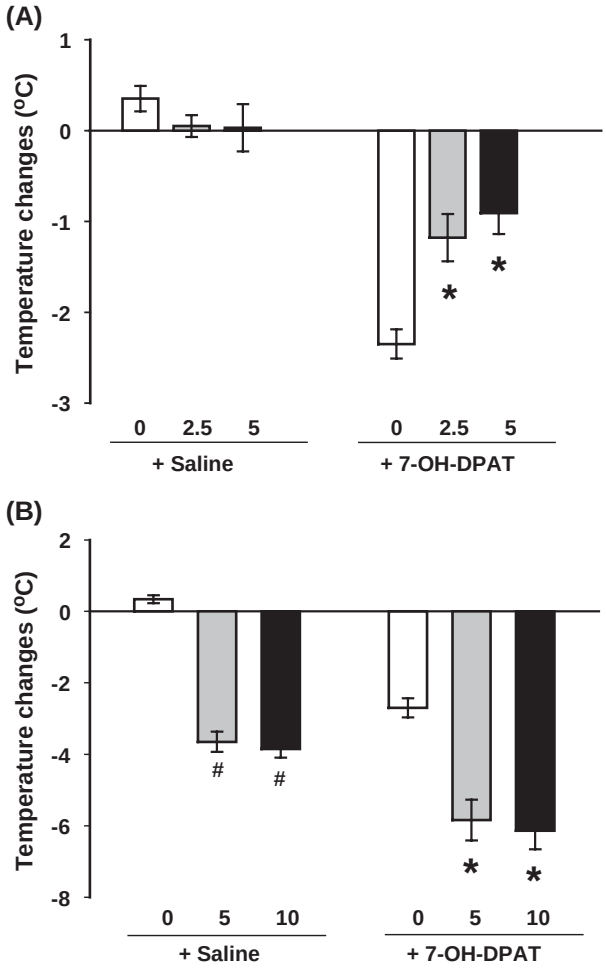

Fig. 7. Inhibitory effect of KKHA-761 on 7-OH-DPAT-induced hypothermia in mice. Rectal temperatures of mice were measured 30 min before and after the injection of 7-OH-DPAT (0.2 mg/kg, s.c.) or saline, and changes of body temperature were calculated. (A) KKHA-761 (2.5 and 5 mg/kg) or (B) clozapine (5 and 10 mg/kg) was administered (i.p.) 30 min before the injection of 7-OH-DPAT or saline. Values were expressed as means ± S.E.M. $^{*}P<0.05$, $^{\#}P<0.05$, compared with corresponding control group, respectively (Dunnett's test).

$P=0.169$], when compared to the vehicle control group (Fig. 6). In contrast, clozapine (1, 2.5 and 5 mg/kg, i.p.) produced a dose-dependent reduction of immobility [$F(3,44)=10.65$, $P<0.001$] in consistent with the report of others (Weiner et al., 2003). A classic antidepressant drug imipramine (25 mg/kg, i.p.) also produced a significant reduction of the immobility time in a model of depression in mice, the forced swimming ($P<0.001$).

3.7. Effects on 7-OH-DPAT-induced hypothermia in mice

Body temperature of the mice was not changed by a single administration (i.p.) of KKHA-761 (2.5 and 5 mg/kg, i.p.) alone, when compared to the saline control group [$F(2,19)=0.776$, $P=0.474$]. However, the selective dopamine D_3 receptor agonist 7-OH-DPAT (0.2 mg/kg, s.c.)-induced hypothermia (-2.35 ± 0.16 °C) was significantly [$F(2,26)=15.22$, $P<0.001$] attenuated by pretreatment of KKHA-761 (2.5 and 5 mg/kg, i.p.) in mice showing -1.18 ± 0.26 ($P<0.05$) and -0.91 ± 0.23 °C ($P<0.05$), respectively (Fig. 7A).

In contrast, treatment with low doses of clozapine (5 and 10 mg/kg, i.p.) alone produced severe hypothermic effects in mice [$F(2,18)=71.7$, $P<0.001$], and furthermore, 7-OH-DPAT-induced hypothermia was markedly enhanced by pretreatment with clozapine 5 or 10 mg/kg [$F(2,21)=16.2$, $P<0.001$], when compared to vehicle-treated control group (Fig. 7B).

3.8. Prolactin secretion in rats

As shown in Fig. 8, serum prolactin level was significantly increased (37.5 ± 2.1 ng/ml, $P<0.05$) by treatment with very high dose of 50 mg/kg (i.p.) of KKHA-761 in rats, when compared to the vehicle treated group (13.5 ± 2.89 ng/ml). However, moderate doses of KKHA-761 (1, 3 and 10 mg/kg, i.p.) produced no significant increases in serum prolactin levels showing 13.4 ± 5.16, 23.5 ± 5.64 or 20.3 ± 5.61 ng/ml, respectively. On the other hand, serum prolactin levels were not influenced by 2.5 and 10 mg/kg (i.p.) of clozapine [$F(2,13)=3.69$, $P>0.05$], whereas prolactin secretion after haloperidol (0.3, 1 and 3 mg/kg, i.p.) was significantly promoted [$F(3,16)=32.9$, $P<0.001$].

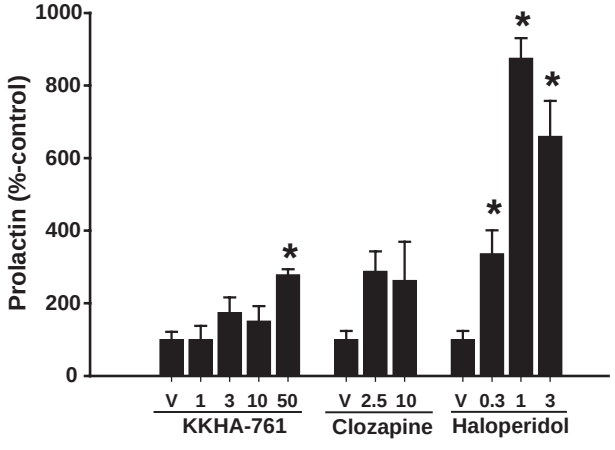

Fig. 8. Effects of KKHA-761, clozapine and haloperidol on serum prolactin secretion in rats. KKHA-761 (1, 3, 10 and 50 mg/kg), clozapine (2.5 and 10 mg/kg), haloperidol (0.3, 1 and 3 mg/kg) or vehicle (V) was administered (i.p.) into the rats, and after 2 h the animals were decapitated and the blood was collected. Plasma concentrations of prolactin were measured using the Biotrak™ rat PRL [^{125}I] assay system. Data were expressed as means± S.E.M. *$P<0.05$, vs. corresponding vehicle-treated group (Dunnett's test).

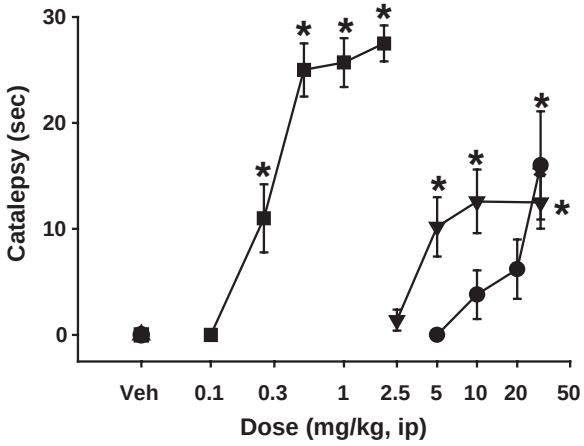

Fig. 9. Effects of KKHA-761, clozapine and haloperidol on induction of catalepsy in mice. KKHA-761 (●), clozapine (▼), haloperidol (■) or vehicle (Veh) was injected (i.p.) into the mice, and 30 or 60 min later, the time that the paws remained on the bar was determined up to a maximum of 30 s. *$P<0.05$, compared with vehicle control group (non-parametric Dunnett's test).

3.9. Catalepsy

KKHA-761 showed an overall significant treatment effect [$H(4)=11.75$, $P=0.019$]. However, there were no significant cataleptic responses in rats at 5, 10 and 20 mg/kg (i.p.), except that 30 mg/kg produced significant catalepsy (16 ± 5.1 s, $P<0.05$) compared with vehicle control group (Fig. 9). Clozapine also produced a small (maximum 12.6 ± 3.0 s) but significant effect [$H(4)=21.2$, $P<0.001$] at the doses of 5–30 mg/kg (i.p.). In contrast, nonselective dopamine antagonist haloperidol potently [$H(5)=47.5$, $P<0.001$] elicited catalepsy even at the relatively low doses (0.25, 0.5, 1 and 2 mg/kg, i.p.).

Table 3
Effects of KKHA-761 and clozapine on rotarod ataxia and acute lethality in mice

Compound	Ataxia	Lethality
	TD_{50} (mg/kg, i.p.)	LD_{50} (mg/kg, i.p.)
KKHA-761	34.4 (31.7~37.2)[a]	243
Clozapine	3.92 (3.2~4.7)	81.5

The KKHA-761 or clozapine was administered (i.p.) 30 min before the testing, and the rotarod deficit (%) was measured by counting the number of animals fallen from the rotating rod within 1 min. The median neurotoxic dose (TD_{50}) was determined as the dose at which 50% of animals showed rotarod deficit. Lethality was scored 48 h after the single administration of the compound, and the median lethal dose (LD_{50}) was determined as the dose which causes the death of 50% of a group of test animals. [a]95% confidence limits.

3.10. Effects on rotarod ataxia and lethality

KKHA-761 did not show any rotarod ataxia at the doses less than 20 mg/kg (i.p.) for 60 min after the treatment (Table 3). However, relatively high doses of 30, 40 and 50 mg/kg (i.p.) of KKHA-761 produced 30%, 70% and 100%-rotarod deficit, respectively. Thus, its median neurotoxic dose (TD_{50}) was calculated to 34.4 mg/kg (95% confidence limits=31.7–37.2 mg/kg). In contrast, rotarod deficit was seen with relatively low doses of clozapine (TD_{50}=3.92 mg/kg, i.p.) in mice.

Single treatment with up to 150 mg/kg (i.p.) of KKHA-761 did not show any death in mice for 1 week, and thus, LD_{50} value of KKHA-761 was 243 mg/kg (i.p.). In contrast, all of the mice treated with more than 150 mg/kg (i.p.) of clozapine died within 48 h, and thus, its LD_{50} was 81.5 mg/kg (Table 3).

4. Discussion

It has been reported that compounds blocking both dopaminergic and serotonergic system may be superior to classical antipsychotics in the treatment of psychiatric disorders (Akunne et al., 2000). The dopaminergic atypical antipsychotic, clozapine, has been shown to be a modulator of 5-HT_{1A}, 5-HT_{2A} or 5-HT_6 receptor (Meltzer, 1995). The potential involvement of 5-HT_{1A} receptor is supported by recent studies showing that activation of 5-HT_{1A} receptors may contribute to clozapine's efficacy against negative symptoms and reduced extrapyramidal side effect liability (Rollema et al., 1997), and the actions at postsynaptic 5-HT_{1A} receptors may be relevant to the therapeutic profile of atypical antipsychotic agents (Newman-Tancredi et al., 2003).

In the present study, KKHA-761 exhibited high affinity binding for DA D_3 receptor labeled with [^{3}H]Spiperone. KKHA-761 showed about 70-fold selectivity for hD_3 (K_i=3.85 nM) and rD_3 receptor (K_i=5.14 nM) over the hD_{2L} receptor (K_i=270.6 nM). KKHA-761 was less active on $hD_{4.2}$ receptor (K_i=154.2 nM) compared with binding potency for hD_3 receptor. KKHA-761 also showed high affinity for cloned human 5-HT_{1A} receptor (K_i=6.4 nM) labeled with [^{3}H]8-OH-DPAT. In contrast, KKHA-761 had only weak or moderate affinities for the human 5-HT_{2A}, 5-HT_{2C}, 5-HT_6 or 5-HT_7 receptors. Furthermore, KKHA-761 retained good selectivity against 27 other receptors, ion channels, and enzymes, showing less than 60% inhibition at higher doses of 10 μM. Although KKHA-761 produced modest activities (≥60% inhibition at 10 μM) on some sites such as α and β adrenoceptors, histamine H_1, sigma σ, sodium channel site 2, considering the very high test concentrations of 10 μM, it is unlikely that KKHA-761 may have IC_{50} values of ≤100 nM. These results, therefore, suggest that KKHA-761 is potent and relatively selective for the dopamine D_3 and serotonin 5-HT_{1A} receptors.

In membranes prepared from CHO cells transfected to express high densities of human D_3 or 5-HT_{1A} receptor, each receptor agonist, dopamine or 5-HT, produced concentration-dependent increases in agonist-stimulated [^{35}S]GTPrS binding. Thus, [^{35}S]GTPrS binding assays have been regarded as an effective method to evaluate the efficacy and potency of agonists and antagonists. Stimulation of D_3 receptor by dopamine enhances [^{35}S]GTPrS binding to G-proteins, and this increase was potently blocked by KKHA-761 with 0.8 μM of IC_{50} value, demonstrating strong antagonist properties at D_3 site. The enhancement of [^{35}S]GTPrS binding by 5-HT at 5-HT_{1A} receptor was also suppressed by KKHA-761. However, it produced only 59% inhibition at 10 μM (IC_{50}=4.3 μM), suggesting relatively weak antagonistic action at 5-HT_{1A} receptor.

Apomorphine-induced climbing behavior is due to the stimulation of dopamine receptors and has been used as a convenient means of in vivo screening dopamine agonists or antagonists (neuroleptics) and to assess striatal dopamine activity (Protais et al., 1976; Costentin et al., 1975). In the present study, apomorphine-induced cage climbing behavior was blocked by intraperitoneal and oral administrations of KKHA-761 without any rotarod ataxia or hyperactivity in mice, and their ID_{50} values were 4.06 and 16.9 mg/kg, respectively. It seems, therefore, that the inhibition of apomorphine-induced climbing behavior by KKHA-761 is due to the selective blockade of dopaminergic receptors because no catalepsy, ataxia or hyperexcitability was observed at the doses tested. In addition, clozapine also significantly blocked the apomorphine-induced climbing. Thus, these results suggest that KKHA-761 has antidopaminergic or antipsychotic activity in mice, and its potency is comparable to that of clozapine.

KKHA-761 did not affect the spontaneous locomotor activity in mice. However, cocaine-induced hyperactivity was completely blocked by low doses of KKHA-761. Cocaine-induced hyperactivity was also inhibited by clozapine in agreement with the previous reports (Merchant et al., 1996; Okuyama et al., 1999; Millan et al., 1998b). Although the antagonism of hyperlocomotion induced by dopamine receptor agonist has been used traditionally to predict antipsychotic efficacy of novel agents (Ogren et al., 1984; Gustafsson and Christensson, 1990), some evidences demonstrate that preferential D_3 antagonists did not block DA agonist-induced hyperactivity at D_3-selective doses (Waters et al., 1993; Griffon et al., 1995), and D_3 receptor-preferring antagonists stimulate locomotor behavior (Manzanedo et al., 1999; Sautel et al., 1995), suggesting that postsynaptic dopamine D_3 receptors exert inhibitory actions on psychomotor functions (Canales and Iversen, 2000; Ahlenius and Salmi, 1994), However, pharmacological studies are not supported by behavioral studies on mice lacking D_3 receptors. The behavioral effects induced by D_3 receptor agonists are identical in wild-type and D_3 receptor-mutant mice (Boulay et al., 1999; Xu et al., 1999; Xu, 1998). In addition, D_3 selective antagonists such as S 18126 and PD152255 also significantly inhibited the DA agonists-induced hyperlocomotion (Millan et al., 1998a; Corbin et al., 1998). The possible reasons for this discrepancy might be their different receptor selectivity as well as procedural differences.

N-methyl-D-aspartate (NMDA) receptor antagonists have psychotomimetic actions in human (Javitt and Zukin, 1991; Snyder, 1980; Jentsch and Roth, 1999; Luby et al., 1959),

suggesting that endogenous dysfunction of NMDA receptor-mediated neurotransmission might contribute to the pathogenesis of schizophrenia (Javitt and Zukin, 1991; Snyder, 1988). Systemic administration of phencyclidine (PCP) or MK801 increases dopamine cell firing rate in the brain (Freeman and Bunney, 1984; French et al., 1985; Murase et al., 1993). In addition, PCP-induced hyperactivity was blocked by 6-hydroxydopamine lesions of the ventral tegmental area (VTA) in rats (French et al., 1985). Furthermore, hyperactivity produced by a low dose of MK801 was dependent upon D_3 receptor stimulation (Leriche et al., 2003). Thus, hyperactivity induced by MK801 can be used as another simple indicating response to assess the in vivo antipsychotic activity of dopamine D_3 receptor-selective drugs (Javitt and Zukin, 1991; Leriche et al., 2003). In the present study, NMDA receptor antagonist MK801 produced hyperlocomotion, in consistent with the reports of others (Wolf et al., 1993; Ouagazzal et al., 1994; Bristow et al., 1993; Leriche et al., 2003), and the MK801-induced hyperactivity in mice was inhibited significantly by KKHA-761, suggesting the antipsychotic activity of KKHA-761 in rats.

Prepulse inhibition (PPI) of acoustic startle in animals is one of the most intensively studied behavioral models with predictive validity for antipsychotic properties of drugs. PPI deficits have been reported in schizophrenic and presumably psychosis-prone subjects (Braff et al., 1992; Simons and Giardina, 1992). The amplitude of the startle reaction is decreased or gated if the main startle stimulus is preceded by the presentation of a weaker stimulus, an occurrence known as PPI (Graham, 1975). KKHA-761 (5 and 10 mg/kg, i.p.) dose-dependently normalized the effect of apomorphine in apomorphine-disrupted PPI in mice, and a significant effect was found at 10 mg/kg. Furthermore, KKHA-761 did not normalize the reduction of startle amplitude induced by apomorphine. Because KKHA-761 (5 and 10 mg/kg) did not produce any rotarod ataxia, catalepsy and an increase in plasma prolactin levels, these results indicate that the effects on PPI are not influenced by nonspecific drug action such as sedation or ataxia. These results support the previous reports that PPI disruptions seen with the selective dopamine agonists may be mediated by central D_3 receptors (Varty and Higgins, 1998; Ellenbroek et al., 2002). However, the present results also raise the question whether receptors other than D_3 play a major role in the above mentioned effects because a selective dopamine D_4 receptor antagonist, NRA0160, clozapine or L-745,870, also significantly reversed the disruption of prepulse inhibition (PPI) in rats produced by apomorphine (Okuyama et al., 1999; Mansbach et al., 1998). In addition, Reavill et al. (2000) reported that selective dopamine D_3 receptor antagonist, SB-277011-A, did not reverse prepulse inhibition deficits in apomorphine- or quinpirole-treated rats, but did significantly reverse the prepulse inhibition deficit in isolation-reared rats. Considering all of these results, the present effects might, at least in part, be caused by serotonin 5-HT_{1A} receptor-dependent mechanism because of the previous reports that the antagonist of 5-HT_{1A} receptors attenuated or abolished the disruptive effects of MK-801 or 8-OH-DPAT on the sensorimotor gating measured in a prepulse-induced inhibition of the acoustic startle response paradigm (Rigdon and Weatherspoon, 1992; Wedzony et al., 2000).

In present study, KKHA-761 did not affect immobility in forced swimming test, a well-validated model to identify potential antidepressants (Porsolt et al., 1978). It has been generally regarded that selective 5-HT_{1A} receptor agonists have pronounced antidepressive and anxiolytic effects and the potency is proportional to its intrinsic activity at 5-HT_{1A} receptors (De Vry, 1995; Koek et al., 2001), supporting the present results that 5-HT_{1A} receptor antagonism by KKHA-761 does not result in antidepressive effects.

KKHA-761 alone did not influence core body temperature in mice. Hypothermia induced by dopamine D_3 receptor agonist 7-OH-DPAT, however, was dose-dependently inhibited by pretreatment with low doses of KKHA-761. In support of these findings, selective D_3 antagonists dose-dependently attenuated induction of hypothermia by 7-OH-DPAT (Millan et al., 1995), indicating that the hypothermic potency of D_3/D_2 agonists correlates with their high affinities at D_3 receptors. Thus, the present results suggest that the potent inhibition of KKHA-761 on 7-OH-DPAT-induced hypothermia may be related with its high affinity at D_3 receptors. In contrast, lower doses of clozapine alone caused hypothermia, and the 7-OH-DPAT-induced hypothermic effect was also potentiated by clozapine, in consistent with the report that dopamine D_4 receptors are not involved in the modulation of body temperature in animals (Millan et al., 1998a).

It has been reported that blockade of dopamine D_2 receptors in striatum underlies the induction of extrapyramidal motor side effects such as catalepsy and rotarod ataxia (Carr et al., 2002). Previous studies also have shown that agents with preferential D_3 or D_4 antagonism lack cataleptogenic effects (Waters et al., 1993; Griffon et al., 1995; Millan et al., 1995). In the present study, KKHA-761 showed a low incidence of catalepsy in mice. Although high dose of 30 mg/kg of KKHA-761 produced about 50% cataleptic response, moderate dose of KKHA-761 (5, 10, 20 mg/kg) did not induce catalepsy. Also the catalepsy induced by clozapine did not exceed 50% even at relatively high dose of 30 mg/kg. In contrast, the nonselective dopamine antagonist haloperidol showed prominent catalepsy at low doses.

Meanwhile, KKHA-761 did not produce any rotarod ataxia at doses up to 20 mg/kg, and thus its rotarod ataxia TD_{50} was 34.4 mg/kg, which is approximately 10-fold higher than that of clozapine. The present results, therefore, demonstrate that KKHA-761 has much lower liability to induce extrapyramidal side effects than clozapine or haloperidol, and KKHA-761 as well as clozapine, unlike haloperidol, has weak dopamine D_2 antagonistic action in vivo.

Antagonism of dopamine D_2 receptor on hypophyseal lactrophs enhance circulating levels of prolactin, whereas the selective dopamine D_3 receptor blockade is unlikely to provoke hyperprolactinaemia (Sokoloff et al., 1992; Ben-Jonathan et al., 1989; McDonald et al., 1984; Nilsson et al., 1996). Although high dose of KKHA-761 (50 mg/kg) significantly increased the serum prolactin levels, moderate doses of KKHA-761 (1, 3 and 10 mg/kg) did not elevate serum prolactin levels in rats. The

preferential D_2 antagonist haloperidol elevated serum prolactin levels at low doses, whereas clozapine did not induce hyperprolactinaemia in rats, in consistent with the previous report (Sokoloff et al., 1992). These results suggest that both KKHA-761 and clozapine did not show any increase in serum prolactin levels at moderate doses, which are much higher than in vivo effective doses obtained in the present study, supporting that selective dopamine D_3 and/or D_4 antagonism is unlikely to provoke prolactin secretion in rats. Furthermore, KKHA-761 was relatively safe in acute lethality test in mice, showing 243 mg/kg of LD_{50} value, compared with that of clozapine.

Taken together, the current results suggest that KKHA-761 is a potent dopamine D_3 receptor antagonist with high 5-HT_{1A} receptor affinity, and has antipsychotic activity with low liability to induce extrapyramidal side effects. Moreover, further pharmacological profiling studies with KKHA-761 could contribute to more precise understanding of the pharmacology and function of the combined dopamine D_3 and serotonin 5-HT_{1A} receptors relevant to neuropsychiatric disorders.

References

Adlersberg M, Arango V, Hsiung S, Mann JJ, Underwood MD, Liu K, et al. In vitro autoradiography of serotonin 5-$HT_{2A}/_{2C}$ receptor-activated G protein: guanosine-5[-(gamma-[^{35}S]thio)triphosphate binding in rat brain. J Neurosci Res 2000;61:674–85.

Ahlenius S, Salmi P. Behavioral and biochemical effects of the dopamine D_3 receptor-selective ligand, 7-OH-DPAT, in the normal and the reserpine-treated rat. Eur J Pharmacol 1994;260:177–81.

Akunne HC, Zoski KT, Davis MD, Cooke LW, Meltzer LT, Whetzel SZ, et al. PD 158771, a potential antipsychotic agent with D_2/D_3 partial agonist and 5-HT_{1A} agonist actions: I Neurochemical effects. Neuropharmacology 2000;39:1197–210.

Ben-Jonathan N, Arbogast LA, Hyde JF. Neuroendocrine regulation of prolactin release. Prog Neurobiol 1989;33:399–447.

Boulay D, Depoortere R, Rostene W, Perrault G, Sanger DJ. Dopamine D_3 receptor agonists produce similar decreases in body temperature and locomotor activity in D_3 knock-out and wild-type mice. Neuropharmacology 1999;38:555–65.

Braff DL, Grillon C, Geyer MA. Gating and habituation of the startle reflex in schizophrenic patients. Arch Gen Psychiatry 1992;49:206–15.

Bristow LJ, Hutson PH, Thorn L, Tricklebank MD. The glycine/NMDA receptor antagonist, *R*-(+)-HA-966, blocks activation of the mesolimbic dopaminergic system induced by phencyclidine and dizocilpine (MK-801) in rodents. Br J Pharmacol 1993;108:1156–63.

Canales JJ, Iversen SD. Dynamic dopamine receptor interactions in the core and shell of nucleus accumbens differentially coordinate the expression of unconditioned motor behaviors. Synapse 2000;36:297–306.

Carlsson A. The current status of the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 1988;1:179–86.

Carr KD, Yamamoto N, Omura M, Cabeza de Vaca S, Krahne L. Effects of the D_3 dopamine receptor antagonist, U99194A, on brain stimulation and D-amphetamine reward, motor activity, and c-*fos* expression in ad libitum fed and food-restricted rats. Psychopharmacology (Berl) 2002;163:76–84.

Corbin AE, Pugsley TA, Akunne HC, Whetzel SZ, Zoski KT, Georgic LM, et al. Pharmacological characterization of PD 152255, a novel dimeric benzimidazole dopamine D_3 antagonist. Pharmacol Biochem Behav 1998;59:487–93.

Costentin J, Protais P, Schwartz JC. Rapid and dissociated changes in sensitivities of different dopamine receptors in mouse brain. Nature 1975;257:405–7.

De Vry J. 5-HT_{1A} receptor agonists: recent developments and controversial issues. Psychopharmacology (Berl) 1995;121:1–26.

Dunham NW, Miya TS, Edwards CD. Pharmacological activity of a series of basic esters mono and dialkyl malonic acid. J Am Pharm Assoc 1957;46:208–9.

Ellenbroek BA, Lubbers LJ, Cools AR. The role of hippocampal dopamine receptors in prepulse inhibition. Eur J Neurosci 2002;15:1237–43.

Freeman AS, Bunney BS. The effects of phencyclidine and *N*-allylnormetazocine on midbrain dopamine neuronal activity. Eur J Pharmacol 1984;104:287–93.

French ED, Pilapil C, Quirion R. Phencyclidine binding sites in the nucleus accumbens and phencyclidine-induced hyperactivity are decreased following lesions of the mesolimbic dopamine system. Eur J Pharmacol 1985;116:1–9.

Graham FK. The more or less startling effects of weak prestimulation. Psychophysiology 1975;12:238–48.

Griffon N, Diaz J, Levesque D, Sautel F, Schwartz JC, Sokoloff P, et al. Localization, regulation, and role of the dopamine D_3 receptor are distinct from those of the D_2 receptor. Clin Neuropharmacol 1995;18:S130–42.

Gustafsson B, Christensson E. Amperozide, a new putatively antipsychotic drug with a limbic mode of action on dopamine mediated behavior. Pharmacol Toxicol 1990;66:12–7.

Ichikawa J, Kuroki T, Kitchen MT, Meltzer HY. *R*(+)-8-OH-DPAT, a 5-HT_{1A} receptor agonist, inhibits amphetamine-induced dopamine release in rat striatum and nucleus accumbens. Eur J Pharmacol 1995;287:179–84.

Javitt DC, Zukin SR. Recent advances in the phencyclidine model of schizophrenia. Am J Psychiatry 1991;148:1301–8.

Jentsch JD, Roth RH. The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 1999;20:201–25.

Kim HS, Park WK. Nitric oxide mediation of cocaine-induced dopaminergic behaviors: ambulation-accelerating activity, reverse tolerance and conditioned place preference in mice. J Pharmacol Exp Ther 1995; 275:551–7.

Kim HS, Rhee GS, Jung JY, Lee JH, Jang CG, Park WK. Inhibition by noncompetitive NMDA receptor antagonists of apomorphine-induced climbing behavior in mice. Life Sci 1996;58:1397–402.

Koek W, Vacher B, Cosi C, Assie MB, Patoiseau JF, Pauwels PJ, et al. 5-HT_{1A} receptor activation and antidepressant-like effects: F 13714 has high efficacy and marked antidepressant potential. Eur J Pharmacol 2001;420: 103–12.

Leriche L, Schwartz JC, Sokoloff P. The dopamine D_3 receptor mediates locomotor hyperactivity induced by NMDA receptor blockade. Neuropharmacology 2003;45:174–81.

Luby ED, Cohen BD, Rosenbaum G, Gottlieb JS, Kelley R. Study of a new schizophrenomimetic drug; sernyl. Arch Neurol Psychiatry 1959;81: 363–9.

Mansbach RS, Brooks EW, Sanner MA, Zorn SH. Selective dopamine D_4 receptor antagonists reverse apomorphine-induced blockade of prepulse inhibition. Psychoparmacology (Berl) 1998;135:194–200.

Manzanedo C, Aguilar MA, Minarro J. The effects of dopamine D_2 and D_3 antagonists on spontaneous motor activity and morphine-induced hyperactivity in male mice. Psychopharmacology (Berl) 1999;143:82–8.

McDonald WM, Sibley DR, Kilpatrick BF, Caron MG. Dopaminergic inhibition of adenylate cyclase correlates with high affinity agonist binding to anterior pituitary D_2 dopamine receptors. Mol Cell Endocrinol 1984; 36:201–9.

Meltzer HY. Role of serotonin in the action of a typical antipsychotic drugs. Clin Neurosci 1995;3:64–75.

Merchant KM, Gill GS, Harris DW, Huff RM, Eaton MJ, Lookingland K, et al. Pharmacological characterization of U-101387, a dopamine D_4 receptor selective antagonist. J Pharmacol Exp Ther 1996;279:1392–403.

Millan MJ. Improving the treatment of schizophrenia: focus on serotonin 5-HT_{1A} receptors. J Pharmacol Exp Ther 2000;295:853–61.

Millan MJ, Peglion JL, Vian J, Rivet JM, Brocco M, Gobert A, et al. Functional correlates of dopamine D_3 receptor activation in the rat in vivo and their modulation by the selective antagonist, (+)-S 14297: 1 Activation of postsynaptic D_3 receptors mediates hypothermia, whereas blockade of D_2 receptors elicits prolactin secretion and catalepsy. J Pharmacol Exp Ther 1995;275:885–98.

Millan MJ, Newman-Tancredi A, Brocco M, Gobert A, Lejeune F, Audinot V, et al. S18126 ([2-[4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)piperazin-1-yl methyl]indan-2-yl]), a potent, selective and competitive antagonist at dopamine D_4 receptors: an in vitro and in vivo comparison with L 745,870 (3-(4-[4-chlorophenyl]piperazin-1-yl)methyl-1H-pyrrolo[2,3b]pyridine) and raclopride. J Pharmacol Exp Ther 1998a;287:167–86.

Millan MJ, Schreiber R, Dekeyne A, Rivet JM, Bervoets K, Mavridis M, et al. S16924 ((*R*)-2-[1-[2-(2,3-dihydro-benzo[1,4] dioxin-5-yloxy)-ethyl]-pyrrolidin-3yl]-1-(4-fluoro-phenyl)-ethanone), a novel, potential antipsychotic with marked serotonin 5-HT_{1A} agonist properties: II Functional profile in comparison to clozapine and haloperidol. J Pharmacol Exp Ther 1998b;286:1356–73.

Millan MJ, Dekeyne A, Rivet JM, Dubuffet T, Lavielle G, Brocco M. S33084, a novel, potent, selective, and competitive antagonist at dopamine D_3-receptors: II Functional and behavioral profile compared with GR218,231 and L741,626. J Pharmacol Exp Ther 2000;293:1063–73.

Murase S, Mathe JM, Grenhoff J, Svensson TH. Effects of dizocipline (MK-801) on rat midbrain dopamine cell activity: differential actions on firing pattern related to anatomical localizaton. J Neural Transm 1993;91:13–25.

Newman-Tancredi A, Cussac D, Audinot V, Pasteau V, Gavaudan S, Millan MJ. G protein activation by human dopamine D_3 receptors in high-expressing Chinese hamster ovary cells: a guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate binding and antibody study. Mol Pharmacol 1999;55:564–74.

Newman-Tancredi A, Rivet JM, Cussac D, Touzard M, Chaput C, Marini L, et al. Comparison of hippocampal G protein activation by 5-HT_{1A} receptor agonists and the atypical antipsychotics clozapine and S16924. Naunyn Schmiedebergs Arch Pharmacol 2003;368:188–99.

Nilsson CL, Ekman A, Hellstrand M, Eriksson E. Inverse agonism at dopamine D_2 receptors Haloperidol-induced prolactin release from GH4C1 cells transfected with the human D_2 receptor is antagonized by *R*(−)-*n*-propylnorapomorphine, raclopride, and phenoxybenzamine. Neuropsychopharmacology 1996;15:53–61.

Ogren SO, Hall H, Kohler C, Magnusson O, Lindbom LO, Angeby K, et al. Remoxipride, a new potential antipsychotic compound with selective antidopaminergic actions in the rat brain. Eur J Pharmacol 1984;102:459–74.

Okuyama S, Kawashima N, Chaki S, Yoshikawa R, Funakoshi T, Ogawa SI, et al. A selective dopamine D_4 receptor antagonist, NRA0160: a preclinical neuropharmacological profile. Life Sci 1999;65:2109–25.

Ouagazzal A, Nieoullon A, Amalric M. Locomotor activation induced by MK-801 in the rat: postsynaptic interaction with dopamine receptors in the ventral stratum. Eur J Pharmacol 1994;251:229–36.

Porsolt RD, Bertin A, Jalfre M. Behavioral despair in rats and mice: strain differences and the effects of imipramine. Eur J Pharmacol 1978;51:291–4.

Protais P, Costentin J, Schwartz JC. Climbing behaviour induced by apomorphine in mice: a simple test for the study of dopamine receptors in striatum. Psychopharmacology (Berl) 1976;50:1–6.

Reavill C, Taylor SG, Wood MD, Ashmeade T, Austin NE, Avenell KY, et al. Pharmacological actions of a novel, high-affinity, and selective human dopamine D_3 receptor antagonist, SB-277011-A. J Pharmacol Exp Ther 2000;294:1154–65.

Rigdon GC, Weatherspoon JK. 5-hydroxytryptamine 1a receptor agonists block prepulse inhibition of acoustic startle reflex. J Pharmacol Exp Ther 1992;263:486–93.

Rollema H, Lu Y, Schmidt AW, Zorn SH. Clozapine increases dopamine release in prefrontal cortex by 5-HT_{1A} receptor activation. Eur J Pharmacol 1997;338:R3–5.

Sautel F, Griffon N, Sokoloff P, Schwartz JC, Launay C, Simon P, et al. Nafadotride, a potent preferential dopamine D_3 receptor antagonist, activates locomotion in rodents. J Pharmacol Exp Ther 1995;275:1239–46.

Seeman P, Van Tol HH. Dopamine receptor pharmacology. Trends Pharmacol Sci 1994;15:264–70.

Simons RF, Giardina BD. Reflex modification in psychosis-prone young adults. Psychophysiology 1992;29:8–16.

Snyder SH. Phencyclidinne. Nature 1980;285:355–6.

Snyder SH. Psychogenic drugs as models of schizophrenia. Neuropsychopharmacology 1988;1:197–9.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC. Molecular cloning and characterization of a novel dopamine receptor D_3 as a target for neuroleptics. Nature 1990;347:146–51.

Sokoloff P, Giros B, Martres MP, Andrieux M, Besancon R, Pilon C, et al. Localization and function of the D_3 dopamine receptor. Arzneimittelforschung 1992;42:224–30.

Varty GB, Higgins GA. Dopamine agonist-induced hypothermia and disruption of prepulse inhibition: evidence for a role of D_3 receptors? Behav Pharmacol 1998;9:445–55.

Waters N, Svensson K, Haadsma-Svensson SR, Smith MW, Carlsson A. The dopamine D_3 receptor: a postsynaptic receptor inhibitory on rat locomotor activity. J Neural Transm 1993;94:11–9.

Wedzony K, Mackowiak M, Zajaczkowski W, Fijal K, Chocyk A, Czyrak A. WAY 100135, an antagonist of 5-HT_{1A} serotonin receptors, attenuates psychotomimetic effects of MK-801. Neuropsychopharmacology 2000;23:547–59.

Weiner I, Schiller D, Gaisler-Salomon I, Green A, Joel D. A comparison of drug effects in latent inhibition and the forced swim test differentiates between the typical antipsychotic haloperidol, the atypical antipsychotics clozapine and olanzapine, and the antidepressants imipramine and paroxetine. Behav Pharmacol 2003;14:215–22.

Wolf ME, White FJ, Hu XT. Behavioral sensitization to MK-801 (dizocilpine): neurochemical and electrophysiological correlates in the mesoaccumbens dopamine system. Behav Pharmacol 1993;4:429–42.

Xu M. Unraveling dopamine D_3 receptor function in response to psychostimulants using a genetic approach. Ann N Y Acad Sci 1998;844:27–39.

Xu M, Koeltzow TE, Cooper DC, Tonegawa S, White FJ. Dopamine D_3 receptor mutant and wild-type mice exhibit identical responses to putative D_3 receptor-selective agonists and antagonists. Synapse 1999;31:210–5.