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Pharmacological evaluation of a novel assay for detecting glycine transporter 1 inhibitors and their antipsychotic potential

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ABSTRACT

Multiple lines of evidence support the notion that hypofunction of glutamatergic neurotransmission is involved in the pathophysiology of schizophrenia. Moreover, glycine and glycine modulators have beneficial effects in patients with schizophrenia, particularly when added on to existing therapy. As glycine is an obligatory co-agonist at the NR1 subunit of the NMDA receptor, blockade of glycine uptake at the glycine transporter type-1 (GlyT1) can enhance low glutamatergic tone. L-687,414 is an antagonist at the glycine modulatory site of the NMDA complex and, behaviorally, increases locomotion. A series of GlyT1 inhibitors along with other psychoactive compounds were examined for their ability to enhance or inhibit the action of L-687,414. GlyT1 inhibitors and the other compounds were examined initially for effects on [^{3}H]-glycine uptake in CHO cells expressing hGlyT1b cDNA and for their ability to displace the NMDA-glycine site ligand [^{3}H]-L-689,560 from isolated rat forebrain membrane preparations. The in vivo activity of these compounds was determined in mice by measuring their ability to prevent L-687,414-induced hyperlocomotion. GlyT1 inhibitors blocked [^{3}H]-glycine uptake in cells expressing the human transporter; other compounds had little or no activity. None of the compounds had affinity for the glycine site of the NMDA receptor complex. Hyperlocomotion induced by L-687,414 was dose-dependently reduced by GlyT1 inhibitors and antipsychotic drugs but not by morphine, fluoxetine or a moderate dose of diazepam. Therefore, this behavioral approach can reliably detect GlyT1 inhibitors which, in turn, may have some activity in common with drugs having antipsychotic effects.

1. Introduction

Deficient glutamatergic neurotransmission originating from N-methyl-D-aspartate (NMDA) receptor hypofunction has been proposed to contribute to the pathophysiology of schizophrenia (Javitt and Zukin, 1991; Olney et al., 1999; Javitt, 2007). This notion is drawn from clinical observations showing that phencyclidine (PCP) or ketamine, non-competitive NMDA receptors antagonists, produce schizophrenia-like psychosis in healthy humans and exacerbate symptoms in patients with schizophrenia. In addition, NMDA receptor antagonists generate negative symptoms and cognitive deficits characteristic of schizophrenia (Allen and Young, 1978; Krystal et al., 1994; Olney and Farber, 1995; Millan, 2005). Thus, much effort has been devoted to identifying mechanisms by which NMDA receptor function could be safely enhanced to counteract the proposed hypoglutamatergia. One such approach has been through the regulation of the high-affinity glycine transporter type-1 (GlyT1). Experimental clinical studies have shown that administration of the GlyT1 inhibitor sarcosine together with standard antipsychotic drugs has been effective in reducing positive and negative symptoms and improving cognitive functions in patients with schizophrenia (Tsai et al., 2004; Lane et al., 2005).

A unique characteristic of the NMDA receptor is that its activation requires, in addition to the binding of the agonist glutamate, the obligatory binding of glycine to the glycine B site that is part of the NMDA receptor (Johnson and Ascher, 1987; Danysz and Parsons, 1998). Thus, synaptic concentrations of glycine in the vicinity of the NMDA receptor can control the strength of glutamatergic transmission. Several sets of molecular, biochemical and behavioral data lead to the view that local concentrations of glycine in the forebrain are tightly regulated by the action of the high-affinity glycine transporter type-1 (GlyT1), the only sodium chloride-dependent glycine reuptake system expressed in this brain region (Harsing et al., 2006; Cubelos et al., 2005). Therefore, one strategy to enhance NMDA receptor function is to elevate glycine concentration in the local microenvironment of synaptic NMDA receptors by inhibition of GlyT1.

In order to develop a behavioral assay that would allow for the rapid identification of GlyT1 inhibitors, it was thought useful to produce a state in which glutamatergic tone would be diminished by some action at the glycine site of the NMDA receptor complex itself. With this in mind, a L-687,414-challenge procedure was

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chosen as L-687,414 is known to inhibit both strychnine-insensitive [^{3}H]-glycine binding to rat cerebral cortex synaptic membranes and glycine-potentiated NMDA responses in cultured cortical neurons (Tricklebank et al., 1994; Leeson et al., 1993). Moreover, L-687,414 produced prominent behavioral effects in mice such as head weaving, body rolling and hyperlocomotion, behaviors similar to those seen after administration of the NMDA antagonist, MK-801 (Tricklebank et al., 1994).

A number of reference GlyT1 inhibitors are now available and some (e.g., ALX5407, ORG24598 and SSR504734) have been shown to elevate glycine in brain extracellular space (Atkinson et al., 2001; Harsing et al., 2006; Depoortere et al., 2005). It was thought that compounds from this class, by increasing glycine and subsequently displacing L-687,414 from its binding site, could attenuate the hyperlocomotion induced by L-687,414. Thus, after the biochemical characterization of a series of GlyT1 inhibitors, the key objective of this study was to investigate L-687,414-induced hyperlocomotion in mice as a new method to assess the in vivo activity of glycine reuptake inhibitors as compared to other psychoactive compounds, particularly drugs with antipsychotic activity.

2. Materials and methods

2.1. Animals

Male NMRI mice (20–30 g) supplied from Iffa Credo, Lyon, France, were housed in a vivarium at controlled temperature (20–22 °C) and a 12 h light/dark cycle (lights on at 6:00 a.m.). Mice were allowed *ad libitum* access to food and water. The experimental procedures used in the present study received prior approval from the City of Basel Cantonal Animal Protection Committee based on adherence to federal and local regulations. Behavioral experiments were conducted during the hours of 8:00 a.m. and 2:00 p.m.

2.2. Drugs

L-687,414 ((3R,4R)-3-amino-1-hydroxy-4-methyl-pyrrolidin-2-one), ALX5407 (R){[3-(biphenyl-4-yloxy)-3-(4-fluoro-phenyl)-propyl]-methyl-amino}-acetic acid , ORG24598 (R){methyl-[3-phenyl-3-(4-trifluoromethyl-phenoxy)-propyl]-amino}-acetic acid, SSR504734 2-chloro-N-((S)-phenyl-(S)-piperidin-2-yl-methyl)-3-trifluoromethyl-benzamide, RO4543338 1-(4-fluoro-phenyl)-8-[2-(4-fluoro-phenyl)-2-hydroxy-cyclohexyl]-1,3,8-triaza-spiro[4.5]decan-4-one (Ceccarelli et al., 2006), RO4840695 1-{4-[4-(2-cyclopropylmethoxy-5-methanesulfonyl-benzoyl)-piperazin-1-yl]-3-fluoro-phenyl}-ethanone (Pinard et al., 2008a), RO4840700 4-[4-(2-cyclopropylmethoxy-5-methanesulfonyl-benzoyl)-piperazin-1-yl]-3-fluoro-benzonitrile (Pinard et al., 2008a), ORG25543, and 4-benzyloxy-N-(1-dimethylamino-cyclopentylmethyl)-3,5-dimethoxy-benzamide were synthesized by the Medicinal Chemistry Department of F. Hoffmann-La Roche. L-687,414, in particular, was prepared following a newly developed synthesis at F. Hoffmann-La Roche (Pinard et al., 2008b). Haloperidol, risperidone and fluoxetine were obtained from Janssen-Cilag, RBI and APIN, respectively. Clozapine, olanzapine, aripiprazole, morphine and diazepam were synthesized at F. Hoffmann-La Roche. Glycine, from Sigma, was dissolved in 0.9% NaCl/0.3% Tween 80 and administered i.p. in a volume of 10 ml/kg body weight. All other drugs were dissolved in water/0.3% Tween 80 and administered orally (except L-687,414, s.c.) in a volume of 10 ml/kg body weight.

2.3. Generation of stable cell line expressing hGLYT1b

The full length cDNAs of human GlyT1b (EMBL S70609) used for expression in eukaryotic cells was amplified by RT-PCR from human brain Poly A$^+$ RNAs (Clontech). The amplified DNA fragments were cloned into pcDNA 3.1 vectors for sequencing. Afterwards, the CDNAs were sub-cloned into pcDNA5/FRT expression vectors (Invitrogen). Flp-in^{TM}-CHO mammalian cells (Invitrogen) containing an integrated Flp Recombinant Target (FRT) site were transfected with the above mentioned expression vectors by a cationic lipid method (Lipofectamine Plus, Invitrogen). After two days in complete HAMs 12 medium, cells were split into fresh medium (maximum 25% confluence) and the selection of clones expressing the genes of interest was performed by addition of Hygromicin B (600 μg/ml medium). Three to five days later, several newly formed foci were manually picked and cells expanded. Single clones were analyzed a few days afterwards for [^{3}H]-glycine uptake and those with highest specific uptake and appropriate kinetic properties were selected.

2.4. [^{3}H]-Glycine uptake

Mammalian CHO cells (Flp-in^{TM}-CHO) stably expressing hGlyT1b cDNA were maintained in monolayer culture at 37 °C in humidified air with 5% CO_2 in Nutrient mixture F-12 containing 10% fetal calf serum, 1% penicillin-streptomycin, 600 μg/ml hygromicin and 1 mM glutamine. On day 1 of the glycine uptake experiments, cells were plated at the density of 40,000 cells/well in complete F-12 medium without hygromycin in 96-well culture plates. On day 2, the medium was aspirated and cells washed twice with uptake buffer (150 mM NaCl, 10 mM Hepes-Tris, pH 7.4, 1 mM $CaCl_2$, 2.5 mM KCl, 2.5 mM $MgSO_4$, 10 mM (+) D-glucose). Thereafter, cells were incubated for 20 min at 22 °C with the experimental compounds. A range of concentrations of the compounds was used to generate data for calculating the concentration of inhibitor resulting in 50% of the effect (i.e, EC_{50}, or the concentration of the competitor inhibiting glycine uptake of 50%). Each concentration effect was tested in quadruplicate. Uptake was started by adding 60 nM [^{3}H]-glycine (15 Ci/mmol, GE Healthcare) and 25 μM of nonradioactive glycine. Nonspecific uptake was determined with 10 μM ORG24598, a potent and specific GlyT1 inhibitor (Brown et al., 2001). Plates were incubated with gentle shaking and the reaction was stopped by aspiration of the mixture and washing three times with ice-cold uptake buffer. After addition of scintillation liquid, the plates were shaken for 3 h and radioactivity was measured by liquid scintillation on a Perkin Elmer TopCount Scintillation plate reader.

The CPM value for each quadruplicate of a concentration of competing compound was averaged ($y1$), then the % specific binding was calculated according to the equation $(((y1 - \text{non-specific})/(\text{total binding} - \text{non-specific})) \times 100)$. The IC_{50} value, defined as the concentration of the compound causing 50% inhibition of specific binding were calculated by linear regression analysis of the dose–response data using an Excel-based computer curve-fitting program.

2.5. Brain membrane preparation

Cerebellum, pons, medulla oblongata and midbrain were dissected out to isolate forebrain which was immediately homogenized in 25 volumes (w/v) of 50 mM Tris–HCl buffer, pH 7.1, containing 10 mM EDTA by Polytron (40–50 s at 10,000 rpm). The crude homogenate was centrifuged at 48,000×g for 10 min at 4 °C. The pelleted homogenate was suspended in the same buffer (25 volumes) as above and incubated 10 min at 37 °C. After an additional centrifugation and suspension in buffer as above, pellets were dissolved in ice-cold 50 mM Tris-acetate, pH 7.1, homogenized by Polytron until a homogeneous suspension could be reached and centrifuged at 48,000×g for 10 min at 4 °C. Final membranes were dissolved in the same buffer to a protein concentration of 0.3 mg/ml, homogenized by Polytron (5 s at 10,000 rpm) and immediately used.

2.6. [^{3}H]-L-689,560 binding (NMDA-glycine site)

Saturation isotherms were determined by addition of ten concentrations of [^{3}H]-L-689,560 (23.63 Ci/mmol Tocris) to rat forebrain membranes (70 μg/well) for 3 h at 4 °C. At the end of incubation, membranes were filtered onto Unifilter 96 well-plate filter plates (GF/B) with a Filtermate 196 harvester (Packard BioScience) and washed three times with cold binding buffer. Nonspecific binding was determined with 20 μM MDL 105,519, a potent and specific NMDA-receptor glycine site antagonist (Baron et al., 1996). Radioactivity on the filters was counted for 3 min on a Topcount microplate scintillation counter (Packard) with quenching correction after addition of 50 μl of Microscint 40. For inhibition experiments, rat forebrain membranes (70 μg/well) were incubated for 1 h at 4 °C with 10 nM [^{3}H] L-689,560 and 6–10 concentrations of experimental compounds (highest concentration, 30 μM). All other experimental conditions were the same as for saturation isotherm determinations.

2.7. Reversal of L-687,414-induced hyperlocomotion in mice

A computerized Digiscan 16 Animal Activity Monitoring System (Omnitech Electronics, Columbus, Ohio) was used to quantify locomotor activity. Data were obtained simultaneously from eight Digiscan activity chambers placed in a soundproof room with a 12 h light/dark cycle. Experiments were performed between 6:30 a.m. and 5:00 p.m. Each activity monitoring chamber consisted of a Plexiglas box (41 × 41 × 28 cm; $W \times L \times H$) with sawdust bedding on the floor surrounded by invisible horizontal and vertical infrared sensor beams. The test boxes were divided by a Plexiglas cross providing each mouse with 20 × 20 cm of moving space. Two animals per cage were monitored simultaneously. Cages were connected to a Digiscan Analyzer linked to a computer that constantly collected the beam status information. The activity detector operates by counting the number of times the beams change from uninterrupted to interrupted status or vice-versa. Records of photocell beam interruptions for individual animals were taken every 5 min over the duration of the experimental session. Initially, a dose–response function for L-687,414 was determined. Thereafter, mice were first treated with compounds administered p.o. or i.p. and, 15 min later, received a s.c. injection of 50 mg/kg of L-687,414. Mice were then transferred from their home cage to the recording chambers for a 15-min habituation phase allowing free exploration of the new environment. Horizontal activity was then recorded for a 60-min time period. As data was not presupposed to be normally distributed, locomotor activity was analyzed using a Kruskal–Wallis ANOVA followed by a Mann–Whitney *t*-test.

For dose–response experiments, the activity value for each group of animals at a given dose of GlyT1 inhibitor or antipsychotic ("*y*1") was expressed as a percent of L-687,414-induced hyperlocomotion and calculated according to the equation (((*y*1 — vehicle horizontal activity)/(L-687,414 horizontal activity — vehicle horizontal activity)) × 100). ID_{50} values, defined as doses of each compound producing 50% inhibition of L-687,414-induced hyperlocomotion, were calculated by linear regression analysis of the dose–response data using an Excel-based computer curve-fitting program.

Table 1
Effects of GlyT1 inhibitors along with haloperidol and aripiprazole on hGlyT1-mediated [^{3}H]-glycine influx. pIC_{50} values are the mean from one experiment performed in quadruplicate.

Compound	Class	pIC_{50}
RO4543338	GlyT1 inhibitor	7.6
RO4840695	GlyT1 inhibitor	7.7
RO4840700	GlyT1 inhibitor	7.8
ORG24598	GlyT1 inhibitor	7.2
ALX5407	GlyT1 inhibitor	7.7
SSR504734	GlyT1 inhibitor	7.1
Haloperidol	Dopamine D2R antagonist (antipsychotic)	4.9
Aripiprazole	Dopamine D2R partial agonist (antipsychotic)	4.9

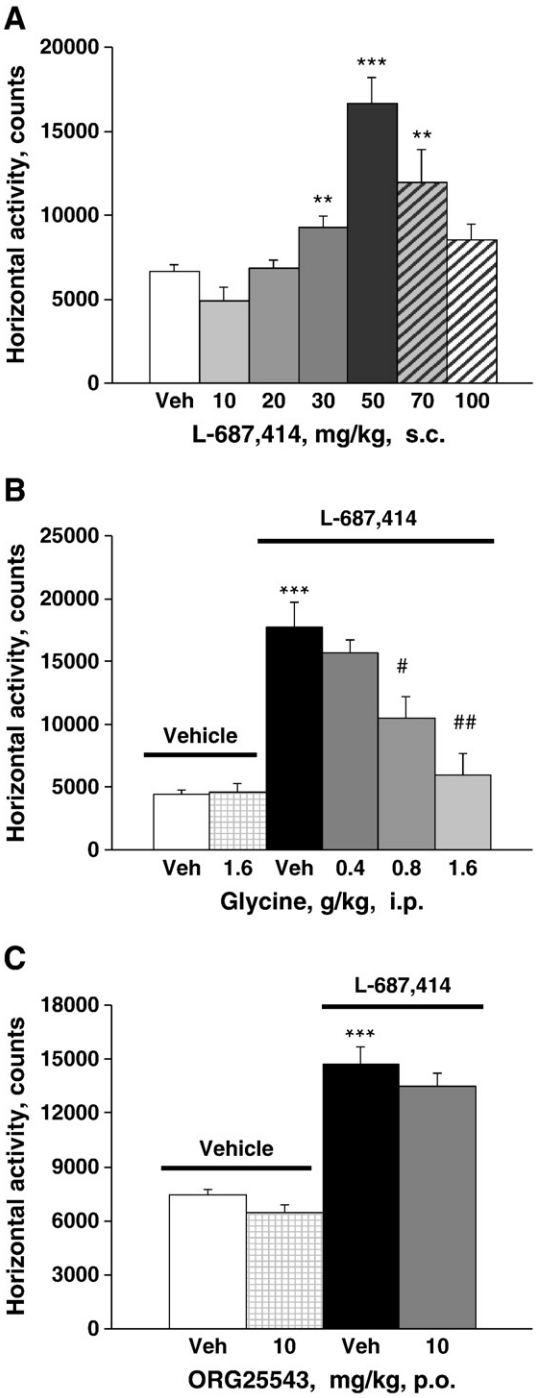

Fig. 1. Panel A: Effect of L-687,414 (10–100 mg/kg, s.c.) on mouse locomotor activity. Mice treated with L-687,414 were recorded for 60 min in a locomotor activity assay. The data represent mean horizontal activity ± S.E.M (n = 7–8 per group). Drug-treated mice displayed significantly more activity than vehicle-treated animals. **, p<0.01, ***, p<0.001 versus vehicle (Veh). Panel B: Dose-dependent inhibition of L-687,414-induced hyperlocomotion by glycine (0.4–1.6 g/kg, i.p.) in mice. The data represent mean horizontal activity counts per group recorded over a 60-min time period; error bars indicate S.E.M. (n = 7–8 per group). ***, p<0.001 versus vehicle (Veh); #, p<0.05, ##, p<0.01 versus L-687,414 alone. Panel C: Lack of effect of the glycine transporter 2 inhibitor ORG25543 (10 mg/kg, p.o.) on L-687,414-induced hyperlocomotion in mice. The data represent mean horizontal activity ± S.E.M. (n = 7–8 per group) recorded over a 60-min time period. ***, p<0.001 versus vehicle (Veh).

3. Results

3.1. [^{3}H]-Glycine uptake

Table 1 shows the effect GlyT1 inhibitors and two antipsychotic drugs on hGlyT1-mediated [^{3}H]-glycine influx. At nanomolar concentrations (pIC_{50} range, 7.1–7.8), all GlyT1 inhibitors inhibited [^{3}H]-glycine uptake in cells expressing the human transporter. Haloperidol and aripiprazole weakly inhibited hGlyT1-mediated [^{3}H]-glycine uptake (pIC_{50}, 4.9, for both compounds). None of the other antipsychotic drugs examined (risperidone, olanzapine and clozapine) nor morphine, fluoxetine, diazepam and ORG25543, a potent GlyT2 inhibitor (Caulfield et al., 2001), significantly affected [^{3}H]-glycine uptake up to 100 μM (data not shown).

3.2. [^{3}H]-L-689,560 binding

To exclude a direct interaction of the compounds with the glycine site of the NMDA receptor complex, compounds were assessed for their ability to displace L-689,560, a potent and selective antagonist at this site, from rat forebrain membranes. The GlyT1 inhibitors as well as the other psychoactive drugs tested showed negligible affinity for the glycine site of the NMDA receptor (data not shown). The affinities of glycine and L-687,414 for the glycine site of the NMDA receptor (pK_i: 6.4 and 6.8, respectively) as well as the dissociation constant for [^{3}H]-L-689,560 (K_d, 6.5 nM) were in accordance with previously reported values (Grimwood et al., 1992). In addition L-687,414, known to bind the strychnine insensitive glycine site of NMDA receptor, was able to displace [^{3}H]-L-689,560 binding from rat forebrain with a pK_i of 6.1 in line with previously published data (Grimwood et al., 1992).

3.3. Effects of L-687,414 on baseline locomotor activity

As compared to vehicle-treated animals, L-687,414 significantly increased locomotor activity over the dose-range of 30–70 mg/kg, s.c. The maximum increase in activity was measured after 50 mg/kg; higher doses resulted in either a weaker or no effect on locomotion (Fig. 1A) and induced loss of righting reflex in some mice. To exclude

Table 2
CEREP selectivity screen undertaken to determine the general pharmacological activity of L-687,414.

Binding assays				Enzyme and cell-based assays	
Target	% control (10 μM)	Target	% control (10 μM)	Target	% control (10 μM)
A_1 (h)	97	M_2 (h)	89	NE uptake	84
A_{2A} (h)	85	M_3 (h)	89	DA uptake	90
A_3 (h)	112	M_4 (h)	96	5-HT uptake	109
α_3 (non-selective)	98	M_5 (h)	105	ATPase (Na^+/K^+)	103
α_2 (non-selective)	96	NK_1 (h)	88	Acetylcholinesterase (h)	96
β_3 (h)	102	NK_2 (h)	95	NE release	107
β_3 (h)	103	NK_3 (h)	119	DA release	101
AT_1 (h)	100	Y_1 (h)	91	5-HT release	84
AT_2 (h)	95	Y_2 (h)	107		
BDZ (central)	97	NT_1 (h) (NTS_1)	92		
BDZ (peripheral)	97	N(neuronal) (a-BTGX-insensitive)	90		
BB (non-selective)	87	N(neuronal) (a-BTGX-sensitive)	84		
B_2 (h)	98	δ (h)	100		
CGRP (h)	82	κ	98		
CB_1 (h)	90	μ (h)	100		
CCK_A (h) (CCK_1)	95	ORL_1 (h)	102		
CCK_B (CCK_2)	105	PACAP (PAC_1)	103		
CRF_1 (h)	97	PCP	70		
D_1 (h)	102	TXA_2/PGH_2 (h) (TP)	111		
D_2 (h)	108	PG_1 (h) (IP)	96		
D_3 (h)	108	P2X	86		
$D_{4.4}$ (h)	95	P2Y	99		
D_5 (h)	98	5-HT_{1A} (h)	103		
ET_A (h)	89	5-HT_{1B} (h)	101		
ET_B (h)	101	5-HT_{2A} (h)	85		
GABA (non-selective)	105	5-HT_{2c} (h)	105		
$GABA_A$	87	5-HT_3 (h)	102		
$GABA_B$	111	5-HT_{5A} (h)	78		
GAL_1 (h)	98	5-HT_6 (h)	86		
GAL_2 (h)	99	5-HT_7 (h)	84		
AMPA	89	σ (non-selective)	92		
Kainate	88	sst(non-selective)	114		
NMDA	96	VIP_A (h) ($VPAC_1$)	98		
Glycine (strychnine sensitive)	96	V_{1A} (h)	104		
Glycine (strychnine insensitive)	**25**	Ca^{2+} channel (L, DHP site)	103		
PDGF	105	Ca^{2+} channel (L, diltiazem site)	105		
IL-8B (h) (CXCR2)	100	Ca^{2+} channel (L, verapamil site)	100		
TNF-α (h)	98	Ca^{2+} channel (N)	98		
CCR_1 (h)	98	K^+_{ATP} channel	92		
H_1 (central)	108	K^+_V channel	98		
H_2	87	SK^+_{Ca} channel	97		
H_3	103	Na^+ channel (site 1)	104		
I_2 (h)	104	Na^+ channel (site 2)	94		
MC_4 (h)	93	SK^-channel	109		
ML_1	99	NE transporter	99		
MAO-A	97	DA transporter	91		
MAO-B	93	GABA transporter7	90		
M_1 (h)	99	5-HT transporter	97		

the possibility that the effect of L-687,414 could be due to off-target activities, its binding activity was tested against 96 binding sites as well as 8 enzyme and cell-based assays and found inactive: i.e., inhibition was less than 30% at 10 μM at these other molecular targets (Table 2). For subsequent behavioral experiments, the 50 mg/kg dose of L-687,414 was selected as the optimal dose to trigger a high and reliable behavioral activation in mice.

3.4. Prevention of L-687,414-induced hyperlocomotion

3.4.1. Effects of glycine and GlyT1 inhibitors

Glycine administered i.p. induced a dose-dependent and significant reduction of L-687,414-induced hyperlocomotion, with an ED_{50} of 0.8 g/kg (Fig. 1B). A nearly complete inhibition was achieved after the highest dose of glycine (1.6 g/kg). This dose of glycine, when administered alone, had no effect on locomotor activity of mice. Unlike glycine, the potent and selective GlyT2 inhibitor, ORG25543, did not alter the hyperlocomotor effects of L-687, 414 (Fig. 1C). In contrast, p.o. administration of all six GlyT1 inhibitors dose-dependently and completely prevented the hyperlocomotor effects of L-687,414. The ID_{50} values were calculated to be 0.6, 1.1, 2.7, 2.9, 3.6 and 4.0 mg/kg for ORG24598, RO4543338, RO4840700, ALX-5407, SSR504734 and RO4840695, respectively. (Fig. 2). When administered alone, the GlyT1 inhibitors did not significantly affect baseline locomotor activity and their effects expressed as percent of vehicle-treated mice were: SSR504734 (30 mg/kg), 69 ± 18%; ALX-5407 (10 mg/kg), 105 ± 11%; RO4840695(30 mg/kg), 82 ± 10%; RO4840700 (30 mg/kg), 113 ± 18%; RO4543338 (10 mg/kg), 104 ± 16%; ORG24598 (10 mg/kg), 112 ± 11%.

3.4.2. Effects of antipsychotic drugs

As shown in Fig. 3, oral administration of haloperidol, clozapine, olanzapine, risperidone and aripiprazole fully prevented L-687,414-induced hyperlocomotion in a dose-dependent manner with ID_{50} values of 0.03, 0.23, 0.04, 0.01 and 0.04 mg/kg, respectively (Fig. 3). At the highest doses tested, these antipsychotic drugs had limited or no effect on locomotor activity when administered alone and their effects expressed as percent of vehicle-treated mice were haloperidol (0.3 mg/kg), 68 ± 10%; clozapine (1 mg/kg), 59 ± 30%; olanzapine (0.3 mg/kg), 99 ± 23%; risperidone (0.03 mg/kg), 103 ± 17%; aripiprazole (1 mg/kg), 53 ± 48%.

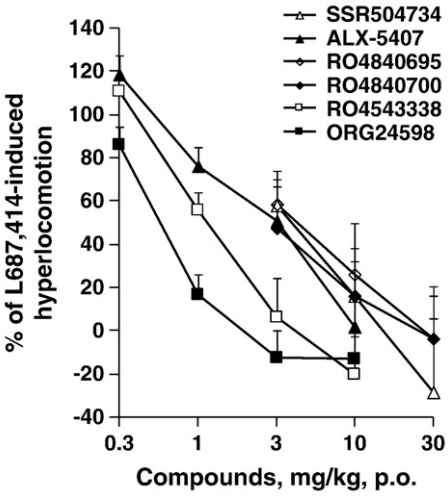

Fig. 2. Dose-dependent inhibition of L-687,414-induced hyperlocomotion by various glycine transporter 1 inhibitors in mice. The data represent the percentage of inhibition of hyperlocomotion induced by 50 mg/kg, s.c., of L-687,414 recorded over a 60-min time period; error bars indicate S.E.M. ($n = 7–8$ per group). When the glycine transporter 1 inhibitors were injected alone, these did not significantly affect the baseline locomotor activity (see Results section).

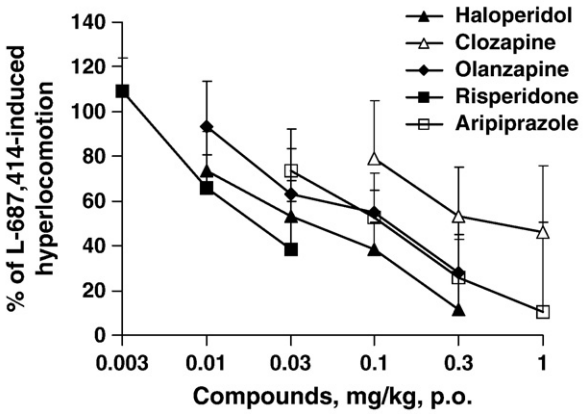

Fig. 3. Dose-dependent inhibition of L-687,414-induced hyperlocomotion by reference antipsychotic compounds in mice. The data represent the percentage of inhibition of hyperlocomotion induced by 50 mg/kg, s.c., of L-687,414 recorded over a 60-min time period; error bars indicate S.E.M. ($n = 7–8$ per group). When the antipsychotic drugs were injected alone, these did not significantly affect baseline locomotor activity (see Results section).

3.4.3. Effects of other psychoactive drugs

Neither the analgesic morphine nor the antidepressant fluoxetine significantly altered L-687,414-induced hyperlocomotion even though at the highest doses studied alone, the compounds decreased locomotor activity. In comparison, the benzodiazepine diazepam, at a dose that alone had effect on locomotion, reduced L-687,414-induced hyperactivity (Fig. 4).

4. Discussion

The primary pharmacological effect of GlyT1 inhibition is an increase in synaptic levels of glycine. In anesthetized rats, microiontophoretic NMDA pulses excite single prefrontal cortex neurons. When these responses were blocked by continuous iontophoretic application of the NMDA receptor glycine-site antagonist, (+)-HA-966, i.v. administration of ALX-5407 reversed this block, likely by a synaptic glycine increase that displaced (+)-HA-966 (Chen et al., 2003). (+)-HA-966, however, may not possess the needed physicochemical properties to elicit marked and reliable behavioral effects when administered systemically to animals. Therefore, it was decided to use L-687,414 as it is structurally related to (+)-HA-966, pharmacologically selective, and exhibits good properties for in vivo research. Moreover, L-687,414 and glycine have similar in vitro potency at the NMDA receptor glycine site, and the former compound produces hyperlocomotion in mice, and has anticonvulsant activity (Tricklebank et al., 1994). In the current study, the locomotor-stimulant effects of L-687,414 were confirmed in NMRI mice.

Hyperlocomotion induced by L-687,414 was blocked by inhibitors of GlyT1 and a series of antipsychotic drugs. These findings substantiate the results of others who have found GlyT1 inhibitors active in other animal procedures thought to predict antipsychotic activity. For example, the GlyT1 inhibitors glycyldodecylamide (GDA; Toth et al., 1986; Javitt et al., 1997), SSR504734, ALX-5407 (NFPS) and ORG24461 (Depoortere et al., 2005; Harsing et al., 2003) as well as glycine (Toth and Lajtha, 1986; Javitt et al., 1997) have been found to attenuate the locomotor hyperactivity induced by acute administration of PCP or MK-801 in rodents. Several groups have also showed that GlyT1 inhibitors reversed or prevented the behavioral deficits induced by repeated administration of PCP in rodents or by ventral hippocampal lesion in neonatal rats (Boulay et al., 2008; Hashimoto et al., 2008; Depoortere et al., 2005; Le Pen et al., 2003; Kato et al., 2001). Together with the current findings in the L-687,414 challenge procedure, this supports the notion that GlyT1 inhibitors may have antipsychotic potential.

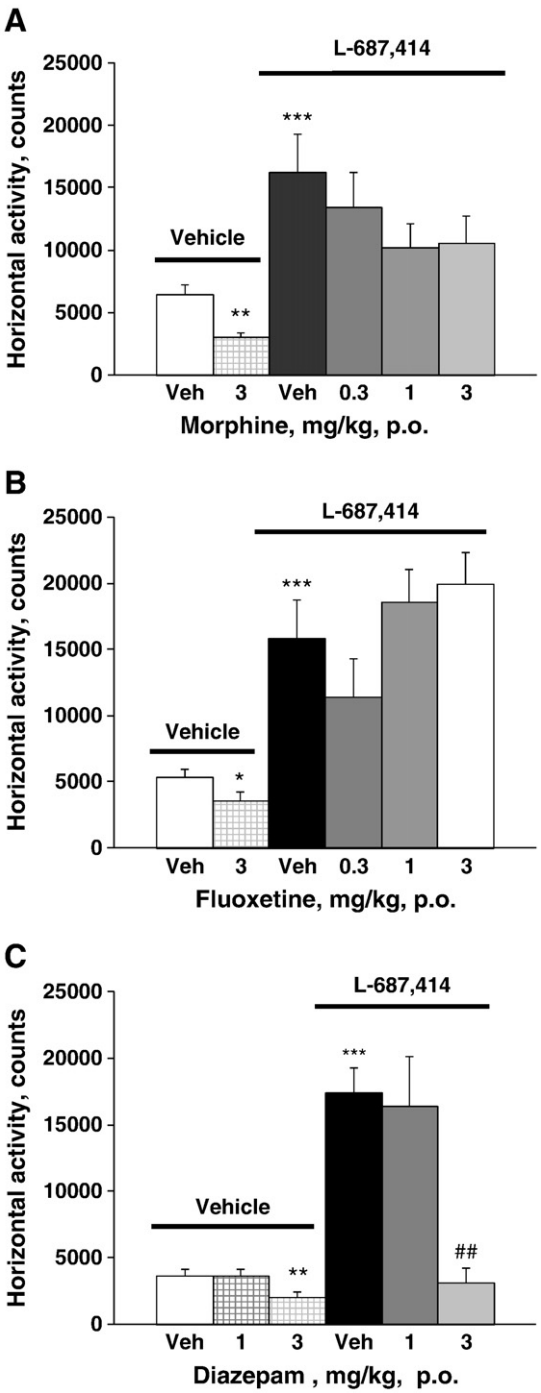

Fig. 4. Effects of a reference analgesic (morphine, 0.3–3 mg/kg, p.o.), a reference antidepressant (fluoxetine, 0.3–3 mg/kg, p.o.) and a reference anxiolytic (diazepam, 1–3 mg/kg, p.o.) on L-687,414-induced hyperlocomotion in mice. The data represent mean horizontal activity counts per group recorded over a 60-min time period, error bars indicate S.E.M. ($n=7$–8 per group). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ versus vehicle (Veh), ##, $p<0.01$ versus L-687,414 alone.

Regarding this theme, there are two reports from clinical research that describe positive outcomes after co-treatment with the weak GlyT1 inhibitor, sarcosine, in patients having schizophrenia. In one study, sarcosine added to stable antipsychotic medicine improved several psychiatric symptoms (Tsai et al., 2004). In a second, patients co-treated with sarcosine had improved PANSS and SANS scores when compared to patients on risperidone monotherapy (Lane et al., 2005). In a single small monotherapy trial, sarcosine had little effect except on the number of patients showing a clinical response (Lane et al., 2008). The partial agonist, D-cycloserine, as co-treatment was generally ineffective on negative symptoms and cognitive impairments in schizophrenics (Buchanan et al., 2007). A number of other exploratory studies, however, with direct-acting agonists, glycine and D-serine, as adjunctive treatment showed clinical improvement (e.g., Javitt et al., 2001; Heresco-Levy et al., 2005).

At doses that had no obvious behavioral effects, glycine and the six potent GlyT1 inhibitors dose-dependently blocked hyperlocomotion induced by L-687,414, confirming the hypothesis that synaptic glycine elevation induced by either direct glycine administration or GlyT1 inhibition can displace L-687,414 from the NMDA receptor binding site and, thus, normalize behavioral alteration induced by NMDA receptor blockade. The observation that in vivo potency of the GlyT1 inhibitors did not correlate well with in vitro potency can possibly be explained by the different pharmacokinetic properties of the compounds. For example, ALX-5407 has higher potency in vitro than ORG24598 yet the latter is active at lower doses in mice. This may be due to the lower brain penetration and poor oral bioavailability of ALX-5407 when compared to the other reference GlyT1 inhibitor, ORG24598 (data not shown). The ineffectiveness of a GlyT2 inhibitor, ORG25543, morphine, fluoxetine and, to a lesser extent, diazepam against L-687,414-induced hyperlocomotion demonstrates the specificity of this behavioral assay and, as ORG25543 had no effect, further suggests that hyperactivity induced by blockade of the NMDA receptor glycine site involves mainly receptors located in the forebrain rather than in the hindbrain or spinal cord.

As antipsychotic drugs have often been shown to attenuate the hyperactivity produced by NMDA receptor open-channel blockers in rodents, and these NMDA antagonists (e.g., PCP) have psychotomimetic effects in humans, such drug-challenge methods in animals are believed to partly mirror some of the symptoms seen in patients with schizophrenia (Mouri et al., 2007). Therefore, it was not surprising to find that haloperidol, olanzapine, risperidone, aripiprazole and clozapine dose-dependently blocked L-687,414-induced hyperlocomotion in the current study. That both antipsychotics and GlyT1 inhibitors altered the behavioral effects of L-687,414 may be explained by the notion that compromised NMDA function by L-687,414 leads to enhanced dopaminergic tone downstream, perhaps in the nucleus accumbens, resulting in hyperlocomotion. An action comparable to this has been previously described for the competitive NMDA antagonist, CPP (Del Arco et al, 2008). Antipsychotic compounds, having dopamine antagonist function, would then attenuate the elevated tone. GlyT1 inhibitors, by acting at the NMDA receptor complex, would have a similar yet indirect action initiated upstream possibly in the prefrontal cortex. Circuity imbalance in which glutamatergic disruption impacts dopamine tone has been associated with schizophrenia (Javitt and Zukin, 1991; Kegeles et al., 2000; Deutsch et al., 2001) and may be further explored in part using the novel behavioral assay described herein with a L-687,414 challenge as it reliably detects the in vivo activity of both antipsychotic drugs and GlyT1 inhibitors.

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