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Glycine transporter (GlyT1) inhibitors with reduced residence time increase prepulse inhibition without inducing hyperlocomotion in DBA/2 mice

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ABSTRACT

Inhibition of the glycine transporter type 1 (GlyT1) leading to potentiation of the glycine site (GlyB) on the *N*-methyl-D-aspartate (NMDA) receptor has been proposed as a novel therapeutic approach for schizophrenia. However, sarcosine-based GlyT1 inhibitors produce undesirable side effects including compulsive walking and respiratory distress. The influence of specific biochemical properties of GlyT1 inhibitors, such as mode of inhibition and residence time, on adverse effects is unknown. Two GlyT1 inhibitors that contain a sarcosine moiety, sarcosine and ALX-5407, and two compounds that do not contain a sarcosine moiety, Roche-7 and Merck (*S*)-13h, were evaluated for their potency, mode of inhibition, and target residence times *in vitro*, and modulation of prepulse inhibition (PPI) and locomotor activity *in vivo*. (*S*)-13h and sarcosine were competitive inhibitors while ALX-5407 and Roche-7 demonstrated mixed noncompetitive inhibition. Potency of GlyT1 inhibition (ALX-5407 > (*S*)-13h > Roche-7 ≫ sarcosine) did not correlate with residence time on GlyT1 (sarcosine = Roche-7 ≪ (*S*)-13h < ALX-5407). ALX-5407 and (*S*)-13h induced compulsive walking, termed obstinate progression (OP), at doses that increased PPI in DBA/2 mice, demonstrating that OP was not a function of mode of inhibition or inhibitor chemotype. Sarcosine and Roche-7 increased PPI without inducing OP, suggesting that compounds with decreased GlyT1 residence time were efficacious without adverse effects. Direct activation of the GlyB site by D-serine did not produce OP. However, OP induced by (*S*)-13h was blocked by strychnine, a glycine receptor (GlyA) antagonist, suggesting that OP induced by GlyT1 inhibition was mediated by GlyA. Thus, GlyT1 inhibitors with short residence times demonstrated efficacy without mechanism-based adverse effects.

1. Introduction

Preclinical and clinical data indicate that *N*-methyl-D-aspartate (NMDA) receptor hypofunction is a primary pathophysiology in schizophrenia [1–3]. Thus, NMDA receptor antagonists mimic the positive, negative, and cognitive symptoms of schizophrenia, exacerbate symptoms in schizophrenics, and can trigger the re-emergence of symptoms in stable patients [4,5]. These findings suggest that enhancing NMDA function may provide therapeutic benefit in schizophrenia. NMDA receptor function can be enhanced by glycine, an obligate coagonist at the strychnine-insensitive (GlyB) site on the receptor channel complex, which acts by increasing the frequency of channel opening [6]. In addition to glycine, D-serine has also been proposed to function as an endogenous coagonist at the GlyB site [7]. Increasing endogenous synaptic glycine concentrations can be achieved by inhibition of the glycine transporter type 1 (GlyT1), which functions to maintain glycine concentrations below saturating levels at the NMDA receptor [8,9]. GlyT1 (SLC6A9) is a Na^+/Cl^--dependent transporter and exists as several isoforms (GlyT1a–d) [9], which cannot be distinguished pharmacologically (unpublished observations). GlyT1 is highly expressed in brainstem and cerebellum and is also present in forebrain regions [10].

As GlyB site agonists and the GlyT1 inhibitors sarcosine and RG1678 improved symptoms in schizophrenics without producing significant adverse effects [2,3,11–14], considerable efforts have been focused on developing GlyT1 inhibitors as a treatment for schizophrenia [15,16]. Preclinically, GlyT1 inhibitors or genetic disruption of neuronal GlyT1 in the forebrain elevated glycine concentration in brain, potentiated NMDA activity in brain slices, and improved prepulse inhibition (PPI) and cognition in behavioral

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models [17–22]. However, the dual role of GlyT1 as the regulator of glycine concentration at both excitatory glutamatergic NMDA and inhibitory glycinergic synapses raises the possibility of side effects associated with GlyT1 inhibition. The strychnine-sensitive glycine receptor (GlyA) and GlyT1 are co-localized in the caudal brainstem, and play a critical role in control of respiration and motor function [23,24]. Consistent with its localization, homozygous GlyT1 deletion in mice was lethal due to motor deficits and respiratory depression, similar to the symptoms of nonketotic hyperglycinemia in humans [23,25]. At doses that elevate brain glycine concentrations, sarcosine-derived GlyT1 inhibitors produced adverse effects in rodents including compulsive, purposeless walking, comparable to obstinate progression (OP) [26], respiratory depression, lateral recumbency, and ataxia [10,16,27]. While the precise mechanism is unknown, it has been suggested that sustained elevation of extracellular glycine in caudal brain areas is causal, raising concerns regarding the safety of GlyT1 inhibitors [10]. It has been postulated that adverse behaviors may arise from GlyT1 inhibitors that contain a sarcosine motif and/or possess a noncompetitive mode of inhibition [28], suggesting that different chemotypes or modification of other properties of the pharmacophore may result in less severe side effects without affecting antipsychotic efficacy.

As preclinical toxicity and efficacy both appear to be associated with GlyT1 inhibition, it remains to be determined whether on-target toxicity can be separated from efficacy. The present study evaluated a series of GlyT1 inhibitors for target potency, mode of inhibition, residence time, activity in the DBA/2 mouse model of PPI [29,30], and the presence of OP in a test of locomotor activity. D-Serine, an NMDA GlyB site agonist, and the weak but selective GlyT1 inhibitor sarcosine were included for comparison.

2. Materials and methods

2.1. Compounds and reagents

ALX-5407, *N*-[(3*R*)-3-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine hydrochloride [31], was obtained from Tocris Bioscience (Ellisville, MO), sarcosine (*N*-methylglycine) and D-serine were from Sigma–Aldrich (St. Louis, MO), and strychnine sulfate was from TCI America (Portland, OR). Merck (*S*)-13h, (*S*)-2-amino-6-chloro-*N*-(1-(4-phenyl-1-(propylsulfonyl)-piperdin-4-yl)ethyl)benzamide [32], and Roche-7, 1-(3-fluoro-4-(4-(2-morpholino-5-nitrobenzoyl)piperazin-1-yl)phenyl)ethanone [33], were prepared by the Medicinal Chemistry group at Cephalon, Inc. as described. [^{3}H]Glycine (52 Ci/mmol) was obtained from PerkinElmer (Shelton, CT). Reagents for the *in vitro* studies, including sodium chloride, potassium chloride, calcium chloride, magnesium chloride, alanine, glycine, TRIS base, and Hybri-max® dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich. HEPES buffer was obtained from Mediatech, Inc. (Herndon, VA).

2.2. Selectivity profiling

Compounds were tested in a panel of selectivity targets according to standard validated protocols under conditions defined by the contractor (MDS Pharma Services, Taipei, Taiwan; http://discovery.mdsps.com). Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Data were expressed as percent inhibition at a compound concentration of 10 μM.

2.3. [^{3}H]Glycine uptake assay for determination of mode of inhibition and potency

Frozen CHO/K1 cells overexpressing recombinant human GlyT1a (CHO-K1/hGlyT1a cells) [34] were thawed and plated at a density of 40,000 cells per well in Costar 3610 (Corning Life Sciences, Corning, NY) 96-well plates and incubated overnight in a humidified cell incubator held at 37 °C with a 10% CO_2 atmosphere. Culture medium (Dulbecco's Modification of Eagle's Medium containing 10% fetal bovine serum, 2 mM glutamine, and 7 μg/mL puromycin) was removed and wells were washed by four exchanges with glycine uptake buffer (120 mM NaCl, 2 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 5 mM alanine, pH 7.5 using 1 M TRIS base), with 250 μL dispensed and 200 μL aspirated with each exchange. Residual wash volume was completely removed from the wells using a very slow aspiration and 80 μL per well of glycine uptake buffer was added. Compounds were diluted by half log steps in DMSO and were added so that the final DMSO concentration was held constant at 0.5% (v/v). After a 30 min incubation, glycine was added to the wells to achieve a final concentration of 30 μM containing 6 μCi/mL [^{3}H]glycine (final specific activity 0.2 μCi/nmol) in an assay volume of 100 μL for IC_{50} value determinations. For mode of inhibition studies, the glycine concentration varied from 0 to 225 μM (final specific activity 0.1 μCi/nmol). Glycine uptake proceeded for 1 h at 27 °C and was stopped by removing unincorporated [^{3}H]glycine and washing as above. Residual wash volume was removed and the cells were lysed with 5 μL of 0.1 N NaOH. Ultima Gold™ scintillation cocktail (PerkinElmer) was added (160 μL per well), plates were sealed, and agitated for 5 min in order to stabilize the signal. Radioactivity was quantitated using a PerkinElmer 1450 Microbeta TriLux™ by counting for 60 s per well.

Specific glycine uptake was calculated as the difference between the total uptake and uptake in presence of 8 mM sarcosine. Data were fit to rate equations describing competitive, noncompetitive, uncompetitive and mixed noncompetitive inhibition in GraFit 5 (Erithacus Software Ltd., Surrey, UK) [35], as well as to double reciprocal plots. Mode of inhibition was determined by selecting the tightest fit (minimized chi squared value) to the theoretical curves.

2.4. [^{3}H]Glycine uptake assay for determination of residence time

Residence time was determined by following the recovery of glycine uptake activity after incubation and subsequent rapid dilution of GlyT1 inhibitors. CHO-K1/hGlyT1a cells were seeded at 44,000 cells per well in 96-well Cytostar T plates (GE Healthcare, Piscataway, NJ) in culture media. After incubation overnight at 37 °C in a humidified, 10% CO_2 atmosphere, culture media was removed from cells and wells were washed by 6 exchanges with glycine uptake buffer on an ELx405 CW™ plate washer (BioTek, Winooski, VT). Compounds were added to cells at a concentration 100-fold above their IC_{50} values for 30 min to ensure complete inhibition of glycine uptake. Cells were washed again and compounds were added at a concentration that was one-tenth of their respective IC_{50} values in order to perturb the established equilibrium. [^{3}H]Glycine was added at 100 μM, 4-fold above the K_m value, at a final specific activity of 0.08 μCi/nmol. Internalization of [^{3}H]glycine (Gly_i) was followed as a function of time (t) using a PerkinElmer 1450 Microbeta TriLux™ by counting for 30 s per well. Data were fit to the rate equation (Eq. (1)) for slow-binding inhibition using Prism 5.01 (GraphPad Software, Inc., San Diego, CA) [36]:

$$Gly_i = C + (v_s \times t) + \frac{v_0 - v_s}{k_{obs}} \times (1 - e^{-k_{obs} \times t}) \quad (1)$$

The steady state velocity (v_s) was constrained in the curve fitting to equal the rate of the uninhibited control, and the initial glycine uptake rate (v_0) was constrained to be >0. The constant (C) represents counts of [^{3}H]glycine that were incorporated just prior

to the first plate reading. Residence time ($t_{1/2}$) was calculated from the determined off rate value (k_{obs}) as defined by Eq. (2):

$$t_{1/2} = \frac{0.69}{k_{obs}} \quad (2)$$

2.5. Animals

Male DBA/2NCrl mice (Charles River Laboratories, Wilmington, MA), were group housed and given food and water *ad libitum*. Mice were on a 12-h/12-h light/dark schedule, with lights on at 7:00 a.m. Mice (18–30 g) were tested once at 7–10 weeks of age during the light phase. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Cephalon, Inc. and were in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals from the U.S. Department of the Health and Human Services.

2.6. Compound administration

ALX-5407 was administered in 25% (2-hydroxypropyl)-β-cyclodextrin (Sigma–Aldrich). (*S*)-13h was administered in 5% DMSO (Sigma–Aldrich)/10% polyethylene glycol 400 (Hampton Research, Aliso Viejo, CA)/85% sterile water (Mediatech) for PPI studies and in 3% DMSO/30% Solutol® HS 15 (BASF, Ludwigshafen, Germany)/67% phosphate buffered saline (Mediatech) for locomotor activity studies. The latter vehicle afforded greater compound solubility but was not used in PPI testing since it increased PPI by itself. Sarcosine was administered in sterile water and Roche-7 in 0.5% methylcellulose (MC, Methocel® A15 Premium LV, Dow Chemical, Midland, MI)/0.2% Tween® 80 (Fisher Scientific, Fair Lawn, NJ)/99.3% sterile water. D-Serine and strychnine were administered in saline (Phoenix Scientific, St. Joseph, MO). Doses of ALX-5407, D-serine and strychnine were calculated using the weight of the free base. GlyT1 inhibitors and D-serine were administered intraperitoneally (i.p.) in a volume of 10 mL/kg. Strychnine was administered subcutaneously (s.c.) at 10 mL/kg.

Doses of ALX-5407, (*S*)-13h, and D-serine were selected based upon literature reports of efficacy of these compounds in PPI [17,32,37–38]. Dose ranges of Roche-7 and sarcosine were initially determined by their *in vitro* affinities for GlyT1 and their brain levels in pharmacokinetic studies. Doses were then iteratively adjusted in the PPI test. Doses of GlyT1 inhibitors for locomotor studies were higher than those for PPI studies since it was initially expected that OP would occur at higher doses than those that were efficacious in PPI. For locomotor testing, doses of ALX-5407 and (*S*)-13h were then adjusted downward.

2.7. Locomotor activity

Open field activity was measured in a modified assay using cylinders instead of square arenas. Mice were placed alone in the center of clear cast acrylic cylinders (40.5 cm outer diameter × 0.316 cm wall thickness × 29.7 cm height) (Ridout Plastics, San Diego, CA). The cylinders were inside Med Associates (St. Albans, VT) clear plastic enclosures (43.2 cm × 43.2 cm × 30.5 cm) with 16 evenly spaced infrared sources and sensors (I/R array) to measure *X* and *Y* coordinates. The enclosures were housed in sound attenuating, ventilated chambers (Med Associates). Low level (7–10 lx), indirect illumination was provided by a 1.8-m rope light kit with 2.5-cm spacing of the lights (18 W, Hampton Bay #361 682, clear, Home Depot, Atlanta, GA), placed around the perimeter of the square enclosure behind the I/R arrays. Horizontal locomotor activity (distance traveled) was monitored in 15-min time blocks. After 1 h of acclimation to the open field arena, mice were briefly removed, administered compounds, and returned to the arena for the remainder of the session. Mice were monitored for 9 h in tests of each GlyT1 inhibitor and D-serine alone and for 5 h in the test of (*S*)-13h and strychnine combined.

2.8. Prepulse inhibition

Mice were tested in SR-LAB™ ABS Startle Reflex Systems (San Diego Instruments, San Diego, CA) using procedures described previously [30]. The time interval between compound administration and testing was dictated by the pharmacokinetic properties of the compounds, and was 2, 4, or 6 h for ALX-5407 and 30 min for D-serine. The PPI test began immediately after administration of (*S*)-13h, sarcosine, and Roche-7. The chamber loudspeaker generated a background white noise of 65 dB for the duration of the 5-min acclimation period and 22-min test session. Following habituation with 5 startle-alone trials (white noise of 120 dB for 40 ms) in block 1, 10 types of trials were given in block 2 in pseudorandom order: prepulse (70, 75, 80, or 85 dB for 10 ms) plus startle (10 trials per prepulse intensity), startle alone (10 trials), no stimulus (10 trials), and prepulse (70, 75, 80, or 85 dB for 10 ms) alone (four trials per prepulse intensity). Prepulse plus startle trials began with the prepulse white noise sound for 10 ms, followed by the startle pulse 100 ms after the offset of the prepulse. Responses were recorded every millisecond for 65 ms after the onset of the startle pulse or of the prepulse in prepulse-alone trials. In no-stimulus trials, baseline activity was recorded for 65 ms. Variable inter-trial intervals ranged from 5 to 25 s, averaging 15 s. Maximum response amplitudes for the 5 startle trials in block 1 and for each of the 10 trial types in block 2 were averaged. Percent PPI at each prepulse sound intensity was defined as [1 − (prepulse plus startle response amplitude/block 2 startle-alone response amplitude)] × 100. Average percent PPI was the mean percent PPI of all 4 prepulse sound intensities.

2.9. Statistics

Behavioral data were analyzed using Student's *t*-tests and analyses of variance (ANOVA) with appropriate within and between subjects designs. *Post hoc* pairwise comparisons versus the vehicle group were made using Bonferroni *t*-tests. When assumptions for parametric tests failed, natural logarithm transformations were performed on startle amplitudes for statistical analyses [40].

3. Results

3.1. Compound potency and mode of inhibition at GlyT1

The mode of inhibition and potency of the four GlyT1 inhibitors were examined in a functional glycine uptake assay (Fig. 1). Kinetic plots of GlyT1 inhibition by (*S*)-13h (Fig. 1A and B) and sarcosine (Fig. 1C and D) showed that these compounds were competitive with respect to the glycine site on the transporter. In contrast, Roche-7 (Fig. 1E and F) and ALX-5407 (Fig. 1G and H) demonstrated mixed noncompetitive kinetics, with effects observed on both the K_m and V_{max} for glycine uptake. In the double reciprocal plots for Roche-7 and ALX-5407, the lines intersected below the *x*-axis indicating that both compounds bound to the transporter–substrate complex. In fact, the derived K_{ii} values for both compounds indicated greater affinity for the transporter–substrate complex than for the transporter alone. Table 1 summarizes the potency and K_{ii} and K_{is} values derived from these plots. IC_{50} values demonstrated a broad range of activity from 0.9 nM for ALX-5407 to 39 μM for

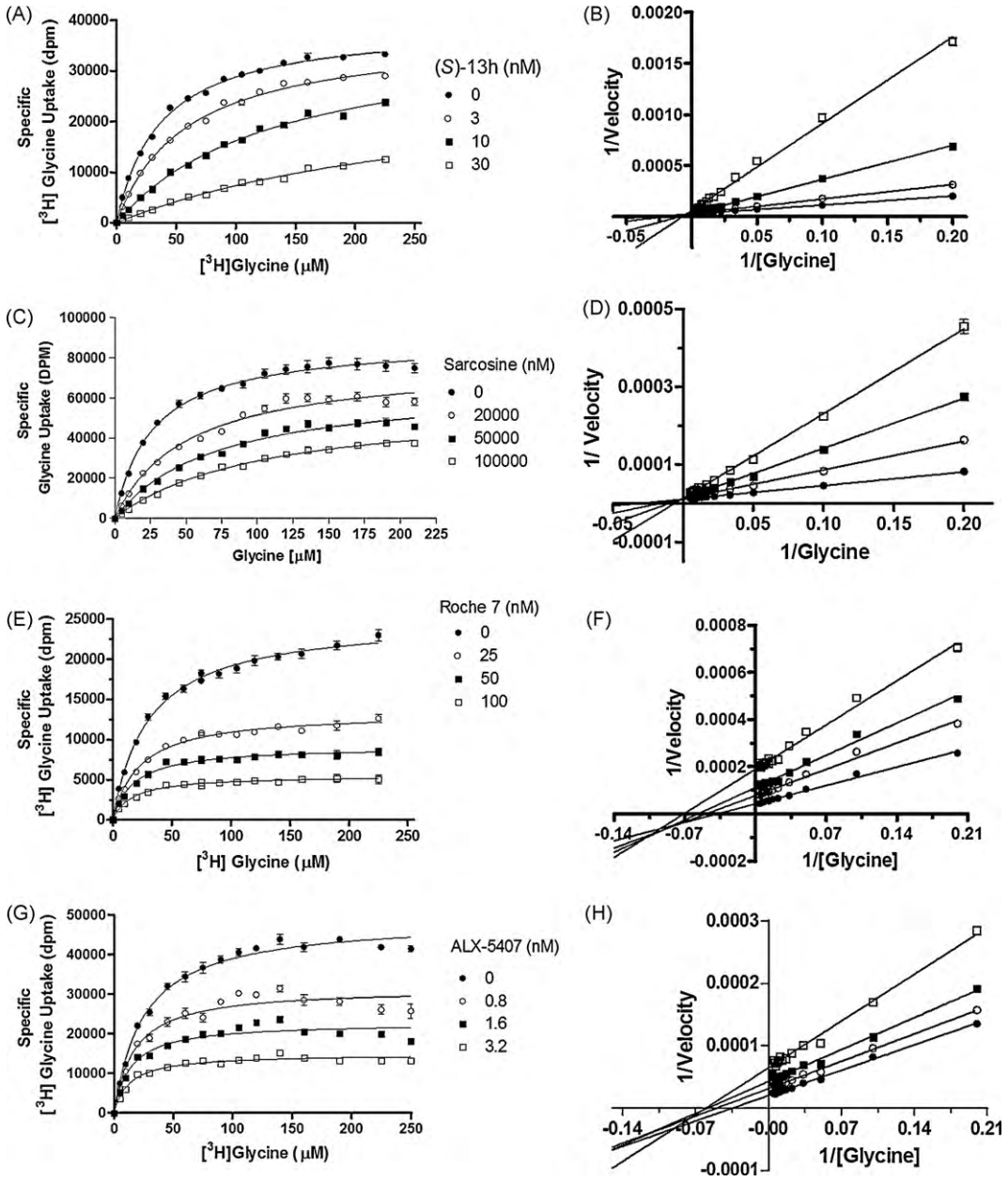

Fig. 1. Mode of inhibition of glycine uptake in CHO-K1/hGlyT1a cells was determined by titrating the indicated glycine concentrations in the absence (closed circles) and presence of GlyT1inhibitors at three concentrations: (*S*)-13h, 3 nM (open circles), 10 nM (closed squares), 30 nM (open squares) (A and B); sarcosine, 20,000 nM (open circles), 50,000 nM (closed squares), 100,000 nM (open squares) (C and D); Roche-7, 25 nM (open circles), 50 nM (closed squares), 100 nM (open squares) (E and F); and ALX-5407, 0.8 nM (open circles), 1.6 nM (closed squares), 3.2 nM (open squares) (G and H). The nonlinear regression plotted for each compound (A, C, E and G) was replotted in double reciprocal form (B, D, F and H). Data points represent the mean ± standard error of the mean (SEM) of six wells from a representative experiment. Data were replicated in 3 or more independent trials.

sarcosine. Thus, sarcosine-derived compounds and compounds lacking a sarcosine moiety representing both competitive and mixed noncompetitive inhibitors were tested for residence time and in behavioral assays.

3.2. Selectivity profiling

In the *in vitro* binding assay of 78 selectivity targets, ALX-5407 (10 μM) produced 54% inhibition of binding to the serotonin

Table 1
Potency and residence time of GlyT1 inhibitors.

Compound	IC_{50} (nM)	K_{is} [K_{ii}] (nM)	Residence time (min)	Mode of inhibition
ALX-5407	0.9 ± 0.2	5.0 ± 1.6 [2.1 ± 0.9]	294 ± 12	Mixed noncompetitive
(*S*)-13h	3.8 ± 1.2	2.3 ± 1.0	103 ± 14	Competitive
Roche-7	25.1 ± 4.8	83.0 ± 7.4 [28.4 ± 1.0]	<10	Mixed noncompetitive
Sarcosine	39,000 ± 5700	13,000 ± 1200	<10	Competitive

Values represent mean ± SEM determined in 3–4 independent trials. K_{is} and K_{ii} values and mode of inhibition were derived from the plots shown in Fig. 1, whereas IC_{50} values were determined at a single glycine concentration as previously reported [34]. Residence times (mean ± SEM) were determined from the data shown in Fig. 2 in three independent trials.

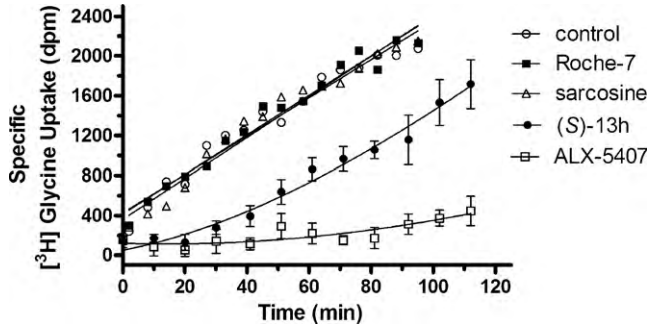

Fig. 2. Residence time of GlyT1 inhibitors varied independently of compound chemotype in CHO-K1/hGlyT1a cells. Cells cultured overnight in 96-well Cytostar plates were treated with the indicated compound and subsequently diluted. [^{3}H]Glycine was then added and recovery of uptake was measured as a function of time for DMSO control (open circles), Roche-7 (filled squares), sarcosine (open triangles), (*S*)-13h (closed circles) and ALX-5407 (open squares). Data points represent the mean ± SEM of six wells from a representative experiment. Data were replicated in 3 or more independent trials.

transporter, 55% inhibition of the neurokinin NK_3 receptor, and 83% inhibition of the serotonin $5HT_{2A}$ receptor. (*S*)-13h, Roche-7 and sarcosine at a concentration of 10 μM did not inhibit binding by >25% to the 78 targets profiled.

3.3. Residence time

Residence time for the four GlyT1 inhibitors was determined by measuring recovery of glycine uptake activity after perturbing the equilibrium between compound and GlyT1 by dilution (Fig. 2). ALX-5407 and (*S*)-13h showed very long-residence times of 294 ± 12 and 103 ± 14 min, respectively (Table 1). In contrast, the recovery of glycine uptake in samples treated with Roche-7 was indistinguishable from the vehicle control, suggesting a short residence time (<10 min). Sarcosine, which competes with glycine as a substrate for GlyT1, also did not show a significant delay in recovery of uptake activity.

3.4. Locomotor activity

Hyperactivity, characterized by compulsive, purposeless walking or obstinate progression, was the earliest observed side effect of GlyT1 inhibition. By using a circular open field arena to prevent in-place running in the corners of a traditional square open field arena [27], OP was quantified as horizontal hyperlocomotor activity (distance traveled). In each study (Figs. 3A–E and 4), mice acclimated to the open field over the first hour ($P < 0.001$). Compound treatment groups did not differ in distance traveled in the first hour.

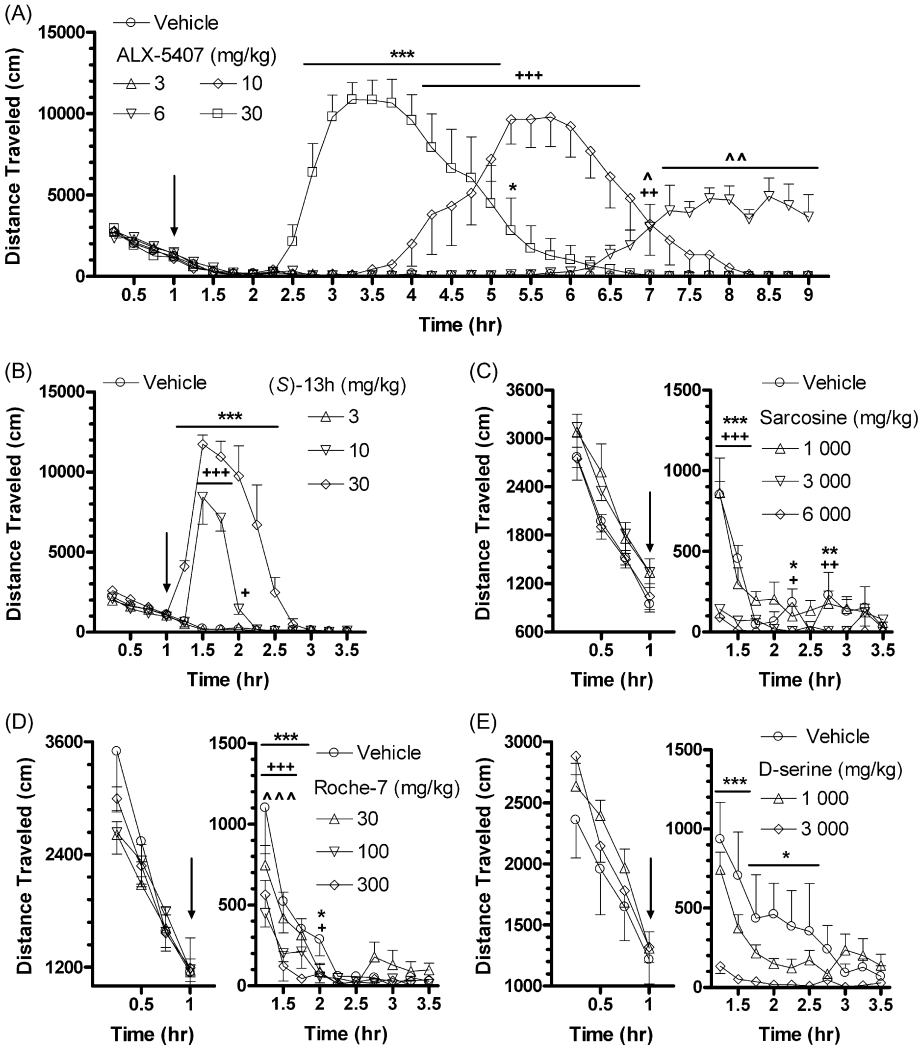

Fig. 3. Locomotor activity in 15-min blocks for ALX-5407 (A), (*S*)-13h (B), sarcosine (C), Roche-7 (D), and D-serine (E). Arrows indicate compound dosing at 1 h. *Post hoc* comparisons versus vehicle for 30 mg/kg ALX-5407 (A), 30 mg/kg (*S*)-13h (B), 6000 mg/kg sarcosine (C), 300 mg/kg Roche-7 (D), and 3000 mg/kg D-serine (E): $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$; for 10 mg/kg ALX-5407 (A), 10 mg/kg (*S*)-13h (B), 3000 mg/kg sarcosine (C), and 100 mg/kg Roche-7 (D): $^+P < 0.05$, $^{++}P \leq 0.01$, $^{+++}P < 0.001$; and for 6 mg/kg ALX-5407 (A) and 30 mg/kg Roche-7 (D): $^\wedge P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$. Locomotor activity for 3 mg/kg ALX-5407 (A) and 3 mg/kg (*S*)-13h (B) overlaid the activity in vehicle-treated mice. Numbers of mice per group = 6, except in A for the vehicle group ($n = 8$) and 6 mg/kg ALX-5407 ($n = 5$). Data points are means ± SEM.

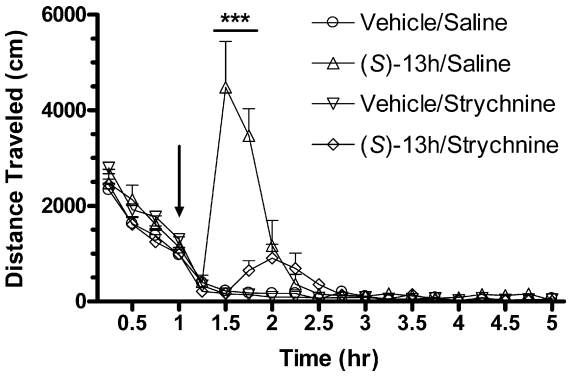

Fig. 4. OP measured as hyperlocomotor activity produced by 10 mg/kg i.p. (*S*)-13h was blocked by 0.4 mg/kg s.c. strychnine. Compounds were administered at 1 h (arrow). The vehicles were: saline for strychnine and 3% DMSO/30% Solutol® (Vehicle) for (*S*)-13h. *Post hoc* all pairwise comparisons indicated that (*S*)-13h/Saline differed from the other 3 treatment groups: $^{***}P < 0.001$. Numbers of mice per group = 9. Data points are means ± SEM.

ALX-5407, a sarcosine-derived, noncompetitive inhibitor (Fig. 1G) with long-residence time on GlyT1 (Fig. 2), was administered at 3, 6, 10, and 30 mg/kg i.p. ALX-5407 produced OP at 6, 10, and 30 mg/kg with a dose-dependent shift in time of onset (Fig. 3A). Mice treated with 3 mg/kg ALX-5407 were indistinguishable from vehicle-treated mice. For 15-min time blocks between 1.25 and 9 h, distance traveled showed a compound treatment effect ($F[4,26] = 31.1$, $P < 0.001$), a time block effect ($F[31,806] = 5.29$, $P < 0.001$), and a compound treatment × time block interaction ($F[124,806] = 11.7$, $P < 0.001$). *Post hoc* analysis of compound treatments versus vehicle at each time block showed that 6, 10, and 30 mg/kg ALX-5407 increased locomotor activity. ALX-5407 at 30 mg/kg increased distance traveled between 2.75 and 5.25 h (2.75–5 h, $P < 0.001$; 5.25 h, $P < 0.01$), 10 mg/kg between 4.25 and 7 h (4.25–6.75 h, $P < 0.001$; 7 h, $P < 0.01$), and 6 mg/kg between 7 and 9 h (7 h, $P < 0.05$; 7.25–9 h, $P < 0.01$). As previously reported [10], following the bout of OP in the 10 and 30 mg/kg ALX-5407 groups, mice showed labored breathing and lateral recumbency.

For the remainder of the compounds (Fig. 3B–E), data for 3.75–9 h are not shown since locomotor activity did not differ from that in vehicle-treated mice. All groups of mice were habituated to the open field arena after 3.75 h and resembled the vehicle-treated mice in the ALX-5407 study (Fig. 3A).

(*S*)-13h, a competitive (Fig. 1A), non-sarcosine GlyT1inhibitor with long residence time (Table 1), was administered at 3, 10, and 30 mg/kg i.p. (*S*)-13h at 10 and 30 mg/kg produced OP, while locomotor activity at 3 mg/kg resembled that of vehicle-treated mice (Fig. 3B). The time of onset of OP was similar for 10 and 30 mg/kg (*S*)-13h, but the overall amount of hyperactivity was dose-dependent. For 15-min time blocks between 1.25 and 9 h, distance traveled showed a compound treatment effect ($F[3,20] = 35.2$, $P < 0.001$), a time block effect ($F[31,620] = 57.577$, $P < 0.001$), and a compound treatment × time block interaction ($F[93,620] = 23.6$, $P < 0.001$). *Post hoc* analysis of compound treatments versus vehicle at each time block showed that 10 and 30 mg/kg (*S*)-13h increased locomotor activity. (*S*)-13h at 30 mg/kg increased distance traveled between 1.25 and 2.5 h ($P < 0.001$) and at 10 mg/kg between 1.5 and 2 h (1.5–1.75 h, $P < 0.001$; 2 h, $P < 0.05$). After 3.5 h, all groups treated with (*S*)-13h did not display the later adverse effects of respiratory distress and lateral recumbency observed with ALX-5407.

Sarcosine, a weak but selective competitive GlyT1 inhibitor (Fig. 1C) that is well tolerated clinically [2,13], was tested to determine whether it would similarly have a low propensity to induce adverse effects in animals. Sarcosine was administered at 1000, 3000, and 6000 mg/kg i.p. Sarcosine at 3000 and 6000 mg/kg decreased locomotor activity (Fig. 3C). For 15-min time blocks between 1.25 and 9 h, distance traveled showed a compound treatment effect ($F[3,20] = 13.9$, $P < 0.001$), a time block effect ($F[31,620] = 12.6$, $P < 0.001$), and a compound treatment × time block interaction ($F[93,620] = 3.44$, $P < 0.001$). *Post hoc* analysis of drug treatments versus vehicle at each time block showed that 3000 and 6000 mg/kg sarcosine decreased locomotor activity between 1.25 and 2.75 h (3000 and 6000 mg/kg, 1.25–1.50 h, $P < 0.001$; 2.25 h, $P < 0.05$; 2.75 h, $P < 0.01$). After 3.5 h, all groups treated with sarcosine did not display any overt side effects.

Roche-7 is a noncompetitive inhibitor (Fig. 1E) that lacks a sarcosine moiety, but has a short residence time similar to sarcosine (Table 1). Roche-7 allowed testing of the hypothesis that adverse effects could be minimized with a short residence time GlyT1 inhibitor, regardless of mode of inhibition, chemotype, and potency. Roche-7 administered at 30, 100, and 300 mg/kg i.p. did not induce OP, and similar to observations with sarcosine, decreased locomotor activity at all doses tested (Fig. 3D). For 15-min time blocks between 1.25 and 9 h, distance traveled showed a compound treatment effect ($F[3,20] = 3.95$, $P = 0.023$), a time block effect ($F[31,620] = 19.3$, $P < 0.001$), and a compound treatment × time block interaction ($F[93,620] = 1.72$, $P < 0.001$). *Post hoc* analysis of compound treatments versus vehicle at each time block showed that 100 and 300 mg/kg Roche-7 decreased distance traveled between 1.25 and 2 h (300 mg/kg, 1.25–1.75 h, $P \leq 0.001$; 2 h, $P < 0.05$ and 100 mg/kg, 1.25–1.5 h, $P < 0.001$; 2 h, $P < 0.05$). At 30 mg/kg Roche-7 decreased locomotor activity at 1.25 h ($P < 0.001$). After 3.5 h, all Roche-7-treated groups did not display the adverse effects observed with ALX-5407.

D-Serine, an agonist of the GlyB site, was tested to determine whether activation of the NMDA receptor mediated the OP behavior observed with ALX-5407 and (*S*)-13h. D-Serine was administered at 1000 and 3000 mg/kg i.p., and at the latter dose decreased locomotor activity (Fig. 3E). For 15-min time blocks between 1.25 and 9 h, distance traveled showed no compound treatment effect, but did show a time block effect ($F[31,465] = 4.40$, $P < 0.001$), and a compound treatment × time block interaction ($F[62,465] = 1.72$, $P < 0.001$). *Post hoc* analysis of compound treatments versus vehicle at each time block showed that 3000 mg/kg D-serine decreased distance traveled between 1.25 and 2.5 h (1.25–1.5 h, $P < 0.001$; 1.75 h, $P < 0.05$; 2 h, $P < 0.01$; 2.25–2.5 h, $P < 0.05$). After 3.5 h, all groups treated with D-serine were similar in locomotor activity to the vehicle group (data not shown).

3.5. Blockade of OP with strychnine

Since activation of the NMDA receptor GlyB site with D-serine did not induce hyperactivity, the OP induced by ALX-5407 and (*S*)-13h was thought to be due to activation of the GlyA receptor by increased glycine levels [10]. To confirm this, the GlyA receptor antagonist strychnine was administered simultaneously with (*S*)-13h. In previous studies, 0.4 mg/kg s.c. strychnine was determined to be subthreshold for the induction of seizure activity or locomotor hyperactivity in DBA/2 mice (data not shown). After 1 h of acclimation in the open field, mice were administered 0.4 mg/kg s.c. strychnine and 10 mg/kg i.p. (*S*)-13h. Whereas mice receiving 10 mg/kg (*S*)-13h and saline displayed OP, those receiving (*S*)-13h and strychnine did not (Fig. 4). Furthermore, mice that were administered (*S*)-13h and strychnine were observed to be indistinguishable from those that received both vehicles. For 15-min time blocks between 1.25 and 5 h, distance traveled showed a compound treatment effect ($F[3,32] = 27.6$, $P < 0.001$), a time block effect ($F[15,480] = 20.6$, $P < 0.001$), and a compound treatment × time block interaction ($F[45,480] = 14.6$, $P < 0.001$). *Post hoc* analysis of compound treatments at each time block showed that 10 mg/kg (*S*)-13h increased locomotor activity

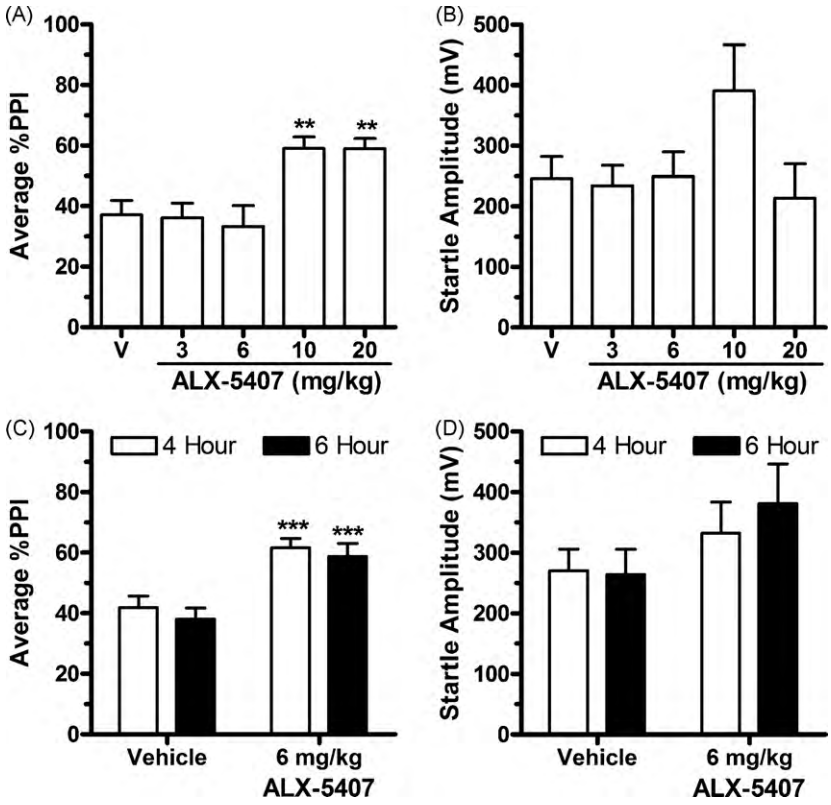

Fig. 5. Average percent PPI (A and C) and startle amplitudes (mV) to 120-dB pulses (B and D) for ALX-5407 tested at 2 h (A and B), 4 h (C and D), and 6 h (C and D) post dosing. *Post hoc* comparisons versus vehicle (V): $^{**}P < 0.01$, $^{***}P < 0.001$. Numbers of mice per group = 20. Bars represent means ± SEM.

in comparison with the other 3 treatment groups at 1.5–1.75 h ($P < 0.001$). Thus, strychnine blocked the locomotor hyperactivity induced by (*S*)-13h.

3.6. Prepulse inhibition

Increased PPI in the DBA/2 mouse model of naturally low PPI has been reported with several GlyT1 inhibitors [17,19,32,41] and was used in the present study to determine efficacious doses of the GlyT1 inhibitors and D-serine. Demonstration of efficacy was particularly important for sarcosine, Roche-7, and D-serine since they did not induce OP and may have similarly not been active in an efficacy test. Because the compound treatment × prepulse intensity interactions were typically not significant and because PPI showed the expected increases as a function of increasing prepulse intensity for each of the compounds, only average percent PPI is illustrated in Figs. 5 and 6.

ALX-5407 was administered at 3, 6, 10, and 20 mg/kg i.p., 2 h before testing. A pretreatment time of 2 h for ALX-5407 in PPI studies was used elsewhere [17,37]. Average percent PPI was increased by compound treatment ($F[4,95] = 7.10$, $P < 0.001$) (Fig. 5A). *Post hoc* comparisons demonstrated an overall increase in percent PPI at 10 and 20 mg/kg ALX-5407 ($P < 0.01$). The startle amplitude during the 120-dB pulse-alone trials was not affected by ALX-5407 (Fig. 5B). The delay in onset of OP with 6 mg/kg ALX-5407 compared with higher doses of ALX-5407 (Fig. 3A) suggested that efficacy in PPI may also be delayed. Thus, 6 mg/kg ALX-5407 was tested in PPI after longer pretreatment times of 4 and 6 h. Although 6 mg/kg ALX-5407 did not increase average percent PPI at 2 h, it did increase average percent PPI at 4 and 6 h (4 h—$F[1,38] = 15.9$, $P < 0.001$ and 6 h—$F[1,38] = 13.5$, $P < 0.001$) (Fig. 5C). At 4 and 6 h, 6 mg/kg ALX-5407 did not alter startle amplitude (Fig. 5D).

(*S*)-13h was administered at 3, 10, and 20 mg/kg i.p., immediately prior to testing and produced a compound treatment effect for average percent PPI ($F[3,36] = 10.9$, $P < 0.001$) (Fig. 6A). In *post hoc* comparisons, average percent PPI was increased by 10 and 20 mg/kg (*S*)-13h ($P < 0.001$). (*S*)-13h did not change startle amplitude during pulse-alone trials (Fig. 6B). Sarcosine was administered at 300, 1000, and 3000 mg/kg i.p., immediately prior to testing. Average percent PPI was increased by sarcosine ($F[3,76] = 6.52$, $P < 0.001$) (Fig. 6C). *Post hoc* comparisons showed that 3000 mg/kg of sarcosine increased average percent PPI compared with vehicle-treated mice ($P < 0.001$). The interaction between compound treatment and prepulse intensity was also significant ($F[9,228] = 2.45$, $P = 0.011$). Sarcosine at 3,000 mg/kg increased percent PPI at prepulse intensities of 70–80 dB (70 dB, $P < 0.001$; 75–80 dB, $P < 0.05$). The sarcosine groups did not differ in startle amplitude (Fig. 6D). Roche-7 was administered at 10 and 30 mg/kg i.p., immediately prior to testing and increased average percent PPI ($F[2,57] = 5.30$, $P = 0.008$) (Fig. 6E). In *post hoc* comparisons, average percent PPI was increased by 30 mg/kg Roche-7 ($P < 0.01$). Roche-7 did not change startle amplitude during pulse-alone trials (Fig. 6F). D-Serine was administered at 100, 300, 1000, and 3000 mg/kg i.p., 30 min prior to testing. Average percent PPI was increased by D-serine ($F[4,95] = 8.60$, $P < 0.001$) (Fig. 6G). In *post hoc* comparisons, average percent PPI was increased by 300, 1000, and 3000 mg/kg of D-serine (300 mg/kg, $P < 0.05$; 1000 and 3000 mg/kg, $P < 0.001$). The startle amplitude during pulse-alone trials was not changed by D-serine (Fig. 6H).

4. Discussion

While the preclinical literature has clearly demonstrated that GlyT1 inhibition elevates extracellular glycine concentration, enhances NMDA receptor-dependent neurotransmission and improves cognition in animal models, identification of adverse effects associated with GlyT1 inhibition has raised concerns regarding safety and has hampered compound development [10,28]. The intent of this study was to compare GlyT1 inhibitors

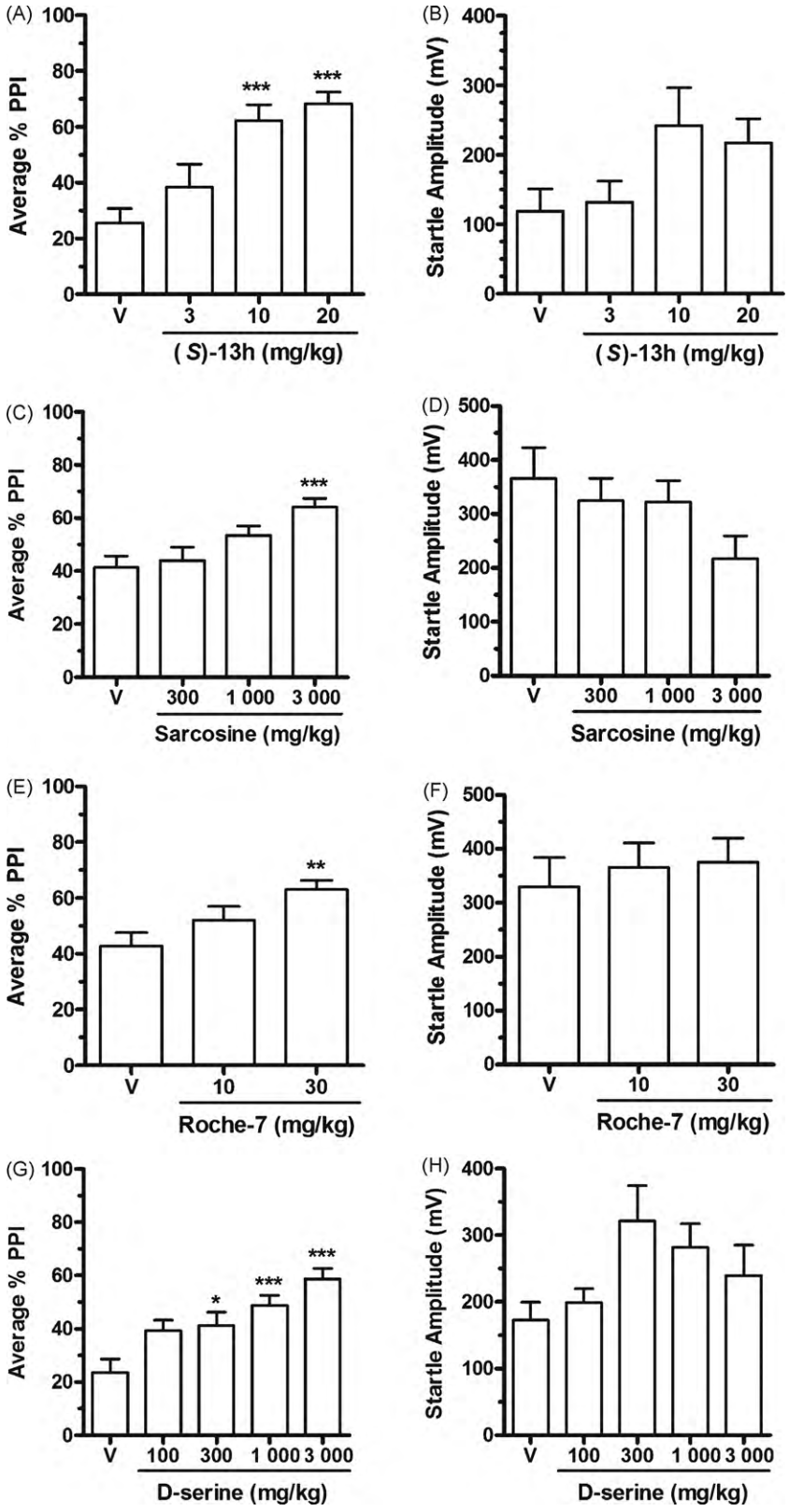

Fig. 6. Average percent PPI (A, C, E, and G) and startle amplitudes (mV) (B, D, F, and H) for (*S*)-13h (A and B), sarcosine (C and D), Roche-7 (E and F), and D-serine (G and H). *Post hoc* comparisons with Bonferroni's t-test versus vehicle (V): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Numbers of mice per group = 20 for sarcosine, Roche-7, and D-serine, and 10 for (*S*)-13h. Because startle amplitude in one mouse was an outlier (Grubb's test), startle amplitude for 20 mg/kg (*S*)-13h was reported in 9 mice. Bars represent means ± SEM.

that differed in chemotype, mode of inhibition, and target residence time and to evaluate the impact of the biochemical profile on ameliorating the obstinate progression associated with GlyT1 inhibition.

ALX-5407 possessed off-target activities at the serotonin transporter, the neurokinin NK_3 receptor, and the serotonin $5HT_{2A}$ receptor, although these activities are relatively weak in comparison to the IC_{50} values for GlyT1 inhibition. It is not known

from this type of profiling whether ALX-5407 acts as an antagonist or an agonist at these receptors, and it cannot be determined whether these activities affected PPI. Selective $5HT_{2A}$ receptor antagonists [42,43] and atypical antipsychotics that possess this activity [44,45] have been reported to increase PPI [17,29,30]. Increased serotonin levels due to transporter inhibition as well as $5HT_{2A}$ receptor agonists, on the other hand, decrease PPI [42,43]. Antagonists of the neurokinin NK_3 receptor have also been suggested as therapeutic targets for schizophrenia, although their activity in PPI has not been studied [46]. However, PPI was increased in DBA/2 mice by a variety of GlyT1 inhibitors [17,19,32,41], including (*S*)-13h, Roche-7, and sarcosine that had no off-target activities. OP was a particularly unusual type of hyperactivity that was not only present after treatment with ALX-5407 but after treatment with other GlyT1 inhibitors [10] (data not shown). Of these other GlyT1 inhibitors, (*S*)-13h had no off-target activities, suggesting that OP was attributable to inhibition of GlyT1 and not to the off-target activities of ALX-5407.

Consistent with the potential antipsychotic efficacy of NMDA receptor activation at the GlyB site, all four GlyT1 inhibitors and D-serine increased PPI in DBA/2 mice. Doses effective in increasing percent PPI for both the GlyT1 inhibitors and D-serine did not alter startle amplitude to the 120-dB pulses alone. Increases in PPI obtained herein were in agreement with previous reports for ALX-5407 and (*S*)-13h, in which ALX-5407 increased PPI at 1 and 10 mg/kg and (*S*)-13h at 3, 30, and 100 mg/kg [17,32]. Additional GlyT1 inhibitors, SSR504734 and SSR103800, have also increased PPI in the DBA/2 mouse model [19,41]. In other rodent models, ALX-5407 increased PPI at 1 mg/kg in C57BL/6 mice treated with the NMDA antagonist MK-801 [37], and ORG 24598 (10 mg/kg) reversed a PPI deficit induced by neonatal lesioning of the ventral hippocampus in rats [18]. GlyB site agonists have not previously been tested in DBA/2 mice but have increased PPI in other rodent models. D-Serine increased PPI in untreated C57BL/6 mice (600 mg/kg [37]) and in ddY mice treated with MK-801 (1800 and 2700 mg/kg [38], 300 and 900 mg/kg [39]). Glycine increased PPI in the neonatal ventral hippocampal-lesioned rat model (800 and 1600 mg/kg) and in ddY mice treated with MK-801 (800 mg/kg) [18,38]. Taken together, these data support the concept that augmentation of synaptic glycine levels increases PPI in a variety of models and may be anticipated to improve impairments involved in schizophrenia.

'Restless running behavior' was first described in mice after administration of 30 mg/kg i.p. ALX-5407 and was thought to be due to an augmentation of NMDA-induced dopamine activity [27]. The motor impairments caused by ALX-5407 were confirmed by Perry et al. [10] and extended to another sarcosine-derived compound, LY2365109. The hallmark of the behavior was a compulsive walking even when confronted with an obstacle, such as the corner of the cage, where the animals continued to walk in place while pushing their heads into the corner. The mice were capable of walking in any direction in the circular arena, and conducted no other exploratory or grooming behavior. This behavior is reminiscent of the syndrome of obstinate progression originally reported in cats with lesions of the nucleus interpeduncularis [26] and in 6-hydroxydopamine lesioned rats after atropine treatment [47]. Similar effects were observed in the present study after dosing with ALX-5407 and (*S*)-13h.

The OP produced by ALX-5407 was striking for the apparent time- and dose-dependence of the effect, which may be due to the pseudo-irreversible nature of ALX-5407 binding to GlyT1 [31,48,49]. Lower doses of ALX-5407 presumably occupied a lower percentage of the available GlyT1, and therefore the rise in glycine concentration required to produce OP was delayed. Consistent with the time delay for onset of OP, 6 mg/kg ALX-5407 was ineffective in PPI when tested 2 h post dosing, but produced efficacy comparable to 20 mg/kg ALX-5407 when tested 4 and 6 h post dosing. The delay in OP of up to 3 h at 10 mg/kg is of interest, as all reports of efficacy in PPI at 10 mg/kg have been reported 2 h post dosing [17,37].

For ALX-5407, the time delay between the generation of efficacy in PPI and the adverse effect of OP provides a plausible explanation as to how such a robust effect as OP did not confound the PPI results in earlier studies [17,37]. For (*S*)-13h, there was a much shorter time delay between efficacy in PPI, tested at 0–30 min after dosing, and the adverse effect of OP, observed at 30–90 min post dosing. These observations suggest that brain glycine concentrations achieved efficacious levels at the time of testing in the PPI assay, but continued to increase, ultimately reaching concentrations that induced OP for both ALX-5407 and (*S*)-13h. It is also important to note that OP and increases in PPI were produced by similar doses (6–20 mg/kg ALX-5407 and 10–20 mg/kg (*S*)-13h), thus indicating no separation between efficacious doses and those causing adverse effect.

The apparent recovery from OP observed over time with 10 and 30 mg/kg ALX-5407 must be interpreted with caution since these mice developed the more advanced symptoms of lateral recumbency and respiratory distress [10,16,27]. Therefore, the return to baseline levels of locomotor activity was not recovery, but a worsening of adverse effect. The adverse effects of respiratory distress and hypotonia were also observed in GlyT1 homozygous knockout mice leading to death on postnatal day 1 [23]. The symptoms in the GlyT1 knockout mice resembled the human condition of nonketotic hyperglycinemia, in which a defect in glycine metabolism leads to high levels of glycine in the central nervous system, respiratory insufficiency and death [25].

Roche-7, sarcosine, and D-serine, while they did not produce OP, did produce hypoactivity at doses similar to or higher than doses that increased percent PPI. Hypoactivity may be seen as a potential side effect. However, sarcosine and D-serine were well-tolerated clinically, and there were no significant adverse effects as compared to placebo, particularly with regard to sedation and fatigue [13,52]. Thus, the hypoactivity observed herein may represent a tolerable effect.

Obstinate progression appeared to be mediated by the action of the elevated glycine concentration acting at the glycinergic receptor, because strychnine, a glycinergic receptor antagonist, inhibited OP induced by (*S*)-13h. However, direct activation of the NMDA receptor with D-serine did not induce OP, suggesting that activation of glutamatergic networks is not involved with OP. These data are in agreement with results reported by Perry et al. [10], where ALX-5407 blocked strychnine-induced elevations in cGMP levels in mouse cerebellum.

Reports of hyperlocomotion after administration of ALX-5407 [27] coupled with the lack of hyperlocomotion after administration of SSR504734, a non-sarcosine, long-residence time, competitive inhibitor [19], suggested that the GlyT1 inhibitor chemotype may impact OP. Furthermore, a compound with a competitive mode of inhibition may be expected to display reduced toxicity since inhibition can be overcome as substrate concentration rises. Specifically, reversible, non-sarcosine-derived inhibitors, such as SSR504734, may be desirable. However, off-target activity at the κ-opiate receptor was observed during selectivity profiling of SSR504734 (data not shown), and this compound was not investigated further. In the present study, OP was induced by (*S*)-13h, a selective, competitive GlyT1 inhibitor that lacks a sarcosine moiety. (*S*)-13h produced peak levels of OP comparable to the sarcosine-derived, mixed noncompetitive inhibitor ALX-5407. The duration of action for (*S*)-13h was shorter, consistent with the reversible nature of its inhibition of GlyT1 and pharmacokinetic profile. This comparison suggests that neither the lack of a sarcosine moiety nor a competitive mode of inhibition is sufficient to ameliorate OP.

Table 2
Summary of biochemical profile of GlyT1 inhibitors.

GlyT1 inhibitor chemotype	Mode of inhibition	
	Competitive	Mixed noncompetitive
Contains sarcosine moiety	**Sarcosine**	ALX-5407
Lacks sarcosine moiety	(*S*)-13h	**Roche**-7

The four GlyT1 inhibitors examined in this study allowed for the following comparisons: 1, compounds that contain a sarcosine moiety and compounds that lack a sarcosine moiety; 2, competitive and mixed noncompetitive inhibitors; and 3, compounds with very short residence time (compounds in bold face) and longer residence time compounds. Sarcosine and Roche-7 increased PPI without inducing OP in DBA/2 mice.

In the present study, OP was induced by GlyT1 inhibitors independently of compound mode of inhibition and chemotype (Table 2) [cf. 28]. Other parameters including potency and compound concentration in brain (data not shown) also did not afford improvement in OP. Because the two compounds in this study that did not induce OP, Roche-7 and sarcosine, are the two less potent compounds, one might think that compound potency against GlyT1 induces OP. However, a proprietary GlyT1 inhibitor with weaker potency than Roche-7 was tested, and this compound also produced OP in our hands. Thus even weak GlyT1 inhibitors are capable of producing OP. In addition, the pharmacokinetic profile of (*S*)-13h indicated that it had the lowest brain concentration and highest clearance of the compounds tested, suggesting that these parameters did not correlate with improved behavior.

Sarcosine and Roche-7, compounds with short residence times, produced only mild hypoactivity, similar to the effect of D-serine, a GlyB site agonist. Thus, of the four compounds tested in the present study, the key mechanistic determinant for amelioration of OP was target residence time. Identification of a set of GlyT1 inhibitors with similar chemotype and mode of inhibition but with a range of residence times would lend additional support to this hypothesis. There was greater than 1000-fold difference in potency between sarcosine and Roche-7, demonstrating that potency did not correlate with residence time for GlyT1 inhibitors and that a potent GlyT1 inhibitor can be efficacious without inducing OP. There is a growing appreciation that target residence time, or the dissociative half-life of the compound–target complex, is an important mechanistic parameter with significant implications for determining both *in vivo* efficacy and adverse effect [50,51]. In particular, target residence time appears to be a key determinant, along with pharmacokinetics, of the *in vivo* duration of action of a compound. Based on the results of the present study, compounds such as ALX-5407 and (*S*)-13h with relatively long target residence times resulted in OP at the same doses that produced efficacy in the PPI model. However, sarcosine and Roche-7, compounds with relatively short target residence times did not produce OP and, in the case of Roche-7, exhibited at least a 10-fold improvement in therapeutic index. Theoretically, the more rapid dissociation of the inhibitor from the transporter may allow glycine to more effectively compete for transport. This may allow for better regulation and maintenance of glycine concentrations in an efficacious range without significantly impacting GlyA receptors. The reason a short residence time GlyT1 inhibitor may differentially impact the two types of synapses, i.e. glutamatergic and glycinergic, is not known. Speculatively, the glycine load may be greater at the glycine receptor after vesicular release than it is at the NMDA receptor, where the glycine concentration is that found in the extracellular milieu. Therefore, it may be possible to mitigate the adverse effects of GlyT1 inhibitors and maintain efficacy by developing a drug-like compound that possesses a short (<10 min) target half-life. Recently, Roche reported the outcome from a Phase II trial with a proprietary compound, RG1678, which was given as an add-on treatment to stably medicated patients with schizophrenia [14] (www.roche.com). RG1678 improved negative symptoms and was well tolerated in these patients. While the residence time of this compound has not been reported, it would be of interest to determine this parameter in light of the short residence time of Roche-7. Future studies with additional compounds possessing a profile similar to Roche-7 and clinical safety studies will be required to fully determine if GlyT1 is a viable antipsychotic target.

Conflict of interest

The authors have no conflicts of interest to report or any involvement to disclose, financial or otherwise, that may bias the conduct, interpretation, or presentation of this work. The authors received compensation as employees from Cephalon, Inc. This research was funded by Cephalon, Inc.

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