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ALX 5407: A Potent, Selective Inhibitor of the hGlyT1 Glycine Transporter

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ABSTRACT

High-affinity glycine transport in neurons and glial cells is a primary means of inactivating synaptic glycine. We have synthesized a potent selective inhibitor of glycine transporter 1 (GlyT1), and characterized its activity using a quail fibroblast cell line (QT6). The glycine transporters GlyT1A, GlyT1B, GlyT1C, and GlyT2 were stably expressed in QT6 cells. The transporters expressed in these cells exhibited appropriate characteristics as described previously for these genes: Na^+/Cl^- dependence, appropriate K_m values for glycine uptake, and appropriate pharmacology, as defined in part by the ability of *N*-methyl glycine (sarcosine) to competitively inhibit glycine transport. Furthermore, the characteristics of the transporters in the cell lines recapitulate the characteristics of glycine transporters observed in tissue preparations. We developed a sarcosine derivative, (*R*)-(*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl])sarcosine (ALX 5407), and examined its activity against the cloned glycine transporters. ALX 5407 completely inhibited glycine transport in the GlyT1 cells, with an IC_{50} value of 3 nM, but had little or no activity at the human GlyT2 transporter, at other binding sites for glycine, or at other neurotransmitter transporters. The inhibition of glycine transport was essentially irreversible. ALX 5407 represents a novel tool in the investigation of *N*-methyl-D-aspartate-receptor function. This class of drug may lead to novel therapies in the treatment of schizophrenia.

Transporters terminate the actions of both glycine and glutamate. Glycine transporters have been cloned and are at present classified into two distinct gene families, glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2). Within the GlyT1 family, at least three splice variants exist, called 1A, 1B, and 1C (Kim et al., 1994). Tissue and cellular expression studies reveal that GlyT1 is distributed widely throughout the CNS, and that distribution correlates better with the localization of *N*-methyl-D-aspartate (NMDA) receptors than with the strychnine-sensitive glycine receptor (Borowsky et al., 1993; Kim et al., 1994; Zafra et al., 1995).

NMDA receptors belong to the family of ionotropic glutamate receptors (Nakanishi et al., 1998). These receptors, named for their sensitivity to the synthetic glutamate-like agonist *N*-methyl-D-aspartate, have been implicated in many physiological and pathophysiological processes, including cognition (Luby et al., 1959; Malhotra et al., 1996), nociception, epilepsy, depression (Trullas and Skolnick, 1990), and schizophrenia (Olney et al., 1999). Because of their apparent obligate role in these conditions, NMDA receptors are an attractive target for therapies that modulate their activity.

NMDA receptors are ligand-gated ion channels that are highly permeable to Ca^{2+} and blocked in a voltage-dependent fashion by Mg^{2+}. The receptors also possess a binding site for the obligatory coagonist glycine, as well as binding sites for polyamines, Zn^{2+}, and redox reagents. Various drugs have been reported to act at these sites, including the psychotomimetic PCP (at the channel site) and D-cycloserine (at the glycine-binding site). NMDA receptors are unusual in that both glutamate and glycine are required for activation (Kleckner and Dingledine, 1988). Therefore, agonism at either site may augment receptor function. For example, glycine enhances NMDA receptor-mediated excitatory postsynaptic potentials in cultured neurons and hippocampal slice preparations (Forsythe et al., 1988; Bergeron et al., 1998), enhances the affinity of glutamate for its binding site (Nguyen et al., 1987), and inhibits desensitization of NMDA receptors (Mayer et al., 1989).

It has been suggested that hypofunction of the glutamatergic system might contribute to some symptoms of schizophrenia (Carlsson and Carlsson, 1990; Javitt and Zukin,

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ABBREVIATIONS: ALX 5407, (*R*)-(*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl])sarcosine; GlyT, glycine transporter; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; QT6, quail fibroblast cell line; CNS, central nervous system; HBS, HEPES-buffered saline; PFC, prefrontal cortex; MDL 105,579, (*Z*)-2-carboxy-4,6-dichloroindole 3-(2′-phenyl-2′carboxy-ene).

1991; Olney and Farber, 1995). Because agonists that act directly on the glutamate-binding site bear the risk of provoking seizures and inducing excitotoxicity, it may be better to augment NMDA receptor activity via indirect mechanisms. Augmenting that activity through the glycine site may be a more desirable strategy for increasing NMDA function than direct glutamate agonists. In humans, glycine has demonstrated efficacy in treating the negative symptoms of schizophrenia in combination with neuroleptic drugs (Waziri, 1988; Heresco-Levy et al., 1996). A weak glycine reuptake inhibitor, glycyldodecylamide, was reported to potently reverse PCP-induced hyperactivity in rats (Javitt and Frusciante, 1997).

Agonism by a neurotransmitter is carefully regulated both by receptor mechanisms and by removal of the agonist. Reuptake mechanisms are essential in terminating the activity of many neurotransmitters. Inhibitors of transporters cause profound physiological effects by increasing the concentration of endogenous receptor agonists. This property of transporters can be exploited pharmacologically, as evidenced by the usefulness of selective serotonin reuptake inhibitors in treating depression. Similarly, a glycine reuptake inhibitor specific for GlyT1 should raise glycine levels and potentiate the actions of the NMDA receptor. We have synthesized a GlyT1-selective antagonist (ALX 5407; Fig. 1), and characterized its activity using a cell line stably transfected with glycine transporter subtypes.

Materials and Methods

Cell Culture and Transfection. QT-6 cells were cultured in Medium 199 with 5% fetal bovine serum, 10% tryptose phosphate, 1% dimethyl sulfoxide, and 1% penicillin/streptomycin. QT-6 cells were transfected with $CaPO_4$ by the following method. Cells were plated at a density of 5×10^5 cells/ml, 10 ml per 100-cm^2 dish. The next day, cells were fed with 9 ml of fresh QT-6 media lacking tryptose phosphate. A DNA cocktail was mixed as follows: 20 mg of target DNA were added to 0.45 ml H_2O and 50 ml of 2.5 M $CaCl_2$ solution. To this was added 500 μl of 2$\times$ balanced salt solution solution (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95). The solution was vortexed immediately and allowed to sit for 20 min at room temperature, at which time a fine precipitate was visible. The solution was added drop-wise to the dish of QT-6 cells. After 24 h, the medium was removed and replaced with fresh complete medium. After a further 24 h, G418 (300 μg/ml final) was added to the medium. After approximately 2 weeks, the cells were fully selected. Dishes were passaged at low density (100–1000 cells per 10-cm^2 dish) and maintained in medium with G418. After 2 weeks, individual colonies were apparent. These colonies were removed with cloning cylinders, and the cell lines were expanded and tested for glycine transport activity.

Transport Assay. Transfected QT-6 cells were plated at 3 to 6 million cells in 96-well dishes previously treated with polylysine. The cells were washed three times with HEPES-buffered saline (HBS; 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 20 mM HEPES, adjusted to pH 7.4 with NaOH). The assay was performed in HBS with 50 nM [^{3}H]glycine (TRK 71; 1 mCi/ml, 18.6 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) and the indicated concentration of inhibitors. The assay was incubated for 20 min at 37°C. Nonspecific uptake was defined with 10 mM unlabeled glycine. The wells were aspirated and washed three times with ice-cold HBS. Scintillant was added to the wells and the plate was counted in a Wallac MicroBeta liquid scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). Protein concentration was quantified using a bicinchoninic acid assay kit (Pierce, Rockford, IL).

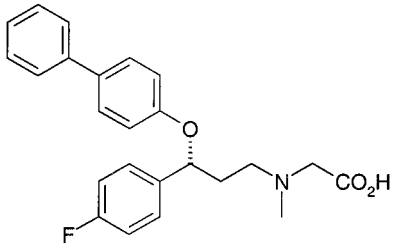


Fig. 1. The chemical structure of ALX-5407.

[^{3}H]MDL 105,519 Binding. Rats were sacrificed and hippocampi and cortices were rapidly dissected on ice. The tissue was homogenized using a Polytron homogenizer (Kinematica AG, Basel, Switzerland) in 20 ml of ice-cold 10 mM Tris-acetate buffer, pH 7.4. The tissue was centrifuged for 30 min at 42,000*g* and resuspended in 20 ml buffer, on ice, for 20 min. The wash and hypotonic lysis steps were repeated 3 times and membranes were stored at −20°C in 100 mg/ml aliquots. On the day of the experiment, membranes were thawed and washed as above three times.

The assay was started with the addition of 4 nM [^{3}H]MDL 105,519 and appropriate concentrations of inhibitors to 90 μl of membranes (10 μg total). The assay was incubated for 30 min at room temperature on an orbital shaker. The reaction was stopped by aspiration onto glass fiber mats with a TomTec filter apparatus. The filter mat was washed three times with ice-cold Tris-acetate buffer, dried in a microwave oven, and sealed in bags. Scintillation cocktail (10 ml, Scintisafe; Fisher Scientific, Pittsburgh, PA) was added and the mats were counted in a Betaplate scintillation counter (PerkinElmer Wallac).

[^{3}H]Strychnine Binding. Rats were sacrificed and spinal columns removed on ice. The vertebrae were cut and the cord was removed and minced with scissors. The cord was homogenized using a Polytron homogenizer for 10 s in 5 mM Tris-acetate buffer (20:1; v/w). The homogenate was centrifuged at 41,000*g* for 30 min. Membranes were resuspended in the same initial volume and lysed for 30 min on ice. The membranes were centrifuged again, and the pellet was stored at −20°C. On the day of the experiment, membranes were thawed, washed, and lysed 5 times. Membranes were then resuspended at a concentration of 2 mg/ml in binding buffer consisting of 25 mM K_3PO_4 and 200 mM KCl, pH 7.4. A 100-μl aliquot was incubated with 10 nM [^{3}H]strychnine and the indicated concentration of blocking drugs for 1 h on ice with shaking. The reaction was stopped by filtration onto GF/C filters soaked in 0.1 mg/ml polylysine and 0.5% bovine serum albumin in phosphate-buffered saline. Filters were washed once with 4 ml of ice-cold binding buffer. Nonspecific binding was defined with either 10 mM glycine or 100 mM strychnine.

Preparation of Aggregates. Rats were sacrificed and brain tissues or spinal columns were dissected on ice. Pooled tissue was forced sequentially through a series of two nylon mesh membranes (Tetko, Inc., Elmsford, NY) in HEPES-buffered saline. The dispersed cells were centrifuged at 2000*g* for 10 min in 50-ml conical tubes. The cells were washed twice more in the same fashion, using HBS. After the final wash, the aggregates were resuspended in HBS, tested for protein concentration, and brought to a concentration of 1 mg/ml in HBS.

Transport Assay (Aggregates). The transport assay using the rat brain or spinal cord aggregates was performed using freshly made aggregates only. The assay was started by adding [^{3}H]glycine and the indicated concentration of inhibitors to 80 μl of aggregates in HBS (final concentration of [^{3}H]glycine, 50 nM; final protein concentration, 100 μg/100 μl). The reaction was incubated for 15 min at 37°C, and stopped by aspiration onto glass fiber mats using a TomTec filter apparatus. The filter mat was washed three times with ice-cold HBS, dried in a microwave, and sealed in bags. Scintillation

cocktail was added, and the mats were counted in a Betaplate scintillation counter (PerkinElmer Wallac).

Microdialysis. Male Sprague-Dawley rats were purchased from Hilltop Farms (Pittsburgh, PA). The rats weighed between 250 and 275 g upon arrival. Animals were group housed in a temperature/humidity (72 ± 5°F/50% ± 5%) controlled vivarium. They were kept on a 12-h light/dark cycle (lights on at 7:00 AM) and allowed food/water ad libitum for 5 to 7 days before cannulations. Under ketamine/xylazine (70%/30%, 100 mg/ml / 20 mg/ml) anesthesia (1.3 mg/kg, i.p.), the animals were implanted with guide cannulae (CMA/Microdialysis, Solna, Sweden) aimed at the prefrontal cortex (anterior/posterior +3.2, medial/lateral ±0.9, dorsal/ventral −1.0, relative to bregma). After implantation, animals were housed individually and allowed 7 days to recover before experimentation.

On the afternoon before experimentation (3:00 PM to 5:00 PM), microdialysis probes [CMA/11 (CMA/Microdialysis); concentric, 3 mm membrane length, 6000-D molecular mass cutoff) were inserted into the guides, and animals were placed in Plexiglas bowels. Artificial cerebrospinal fluid (147 mM NaCl, 2.4 mM KCl, 1.2 mM $CaCl_2$, 0.9 mM $MgCl_2$) was perfused through the probes at 0.2 μl/min overnight to allow the animals to acclimate to the probes and the chambers. On the morning of experimentation, the flow rate was increased to 2.0 μl/min. The experiment consisted of 2 h of baseline sample collection followed by at least 90 min of postinjection sample collection. Samples were collected in 30-min intervals. After completion of each experiment, animals were euthanized with CO_2, and brains were removed and sectioned for histological verification of probe placement.

Samples were analyzed for glycine content using a Waters Alliance 2690 Separations Module System, a Waters 474 Scanning Fluorescence Detector (5-μl flow cell; excitation wavelength, 260 nm; emission wavelength, 455 nm), and Waters Millennium 32 Software (all from Waters, Milford, MA). Mobile phase [A, 900 ml of 0.5 M NaH_2PO_4 (8.7548 g), 240 ml of MeOH, 20 ml of acetonitrile, 10 ml of tetrahydrofuran (stored under N_2), pH adjusted to 6.2 with NaOH (10N); B, 670 ml of 0.2 M NaH_2PO_4 (3.283 g), 555 ml of MeOH, 30 ml of tetrahydrofuran, pH adjusted to 6.2 with NaOH (10N)] was delivered using a gradient paradigm at 0.5 ml/min. Samples underwent precolumn derivatization using fluoraldehyde (5-μl sample/5 μl of fluoraldehyde; 1.5 min mixing delay; Pierce Chemical). The column was preceded by a Waters μBondapak C18 precolumn. The column consisted of a Waters Spherisorb ODS2, C18, 5 μm, 4.6 × 250 mm, maintained at 30°C. Chromatograms were analyzed by measurement of area under the glycine peak and corrected for concentration by comparison to area under the standard glycine peak.

All drugs were administered orally via 18-gauge gavage needles in a volume of 4 ml/kg. Doses refer to weight of free base and all drugs were dissolved in a 30% propylene glycol/H_2O solution (v/v). Propylene glycol was purchased from Sigma Chemical (St. Louis, MO). Test agents were supplied by the Department of Chemistry at Allelix Neuroscience Inc. (Cranbury, NJ). Two-way analysis of variance with repeated measures was used to analyze differences between vehicle and treatment groups over time and across individual time points. The experiments in this study were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Results

Pharmacology of Glycine Transport in Aggregates from Rat Brain and Spinal Cord. In the rat, GlyT1 is expressed diffusely throughout the CNS (excluding the spinal cord), whereas GlyT2 is expressed primarily in pons, cerebellum, and spinal cord, which corresponds well to the known localization of strychnine-sensitive glycine receptors. We used this differential expression pattern to ascertain whether ALX 5407 inhibits glycine transport by a GlyT1- or GlyT2-dependent manner, using cell aggregates prepared from rat brain or spinal cord. [^{3}H]Glycine uptake in aggregates prepared from rat brain was blocked by coadministration of *N*-methyl glycine (sarcosine), a known inhibitor of the GlyT1 transporter (Fig. 2A) with an IC_{50} value of 13 μM. Sarcosine had no effect on uptake activity in spinal cord aggregates (Fig. 2B). [^{3}H]Glycine uptake in each preparation was diminished in the presence of increasing concentrations of unlabeled glycine. ALX 5407 completely blocked [^{3}H]glycine uptake in rat brain aggregates with an IC_{50} value of 3 nM (Fig. 3A) but had little effect on uptake in spinal cord aggregates up to concentrations of 100 μM (Fig. 3B). Specific transport was typically 80% of total radioactivity.

Expression of Glycine Transporter Subtypes in Quail Fibroblast Cells. The cDNA for the hGlyT1 isoforms 1A, 1B, and 1C were obtained from the laboratory of Dr. Marc Caron (Duke University, Durham, NC), and ligated into pcDNA3 expression vectors. The human homolog of the GlyT2 transporter was cloned previously at Allelix Neuroscience (Albert et al., 1999), and was also ligated into

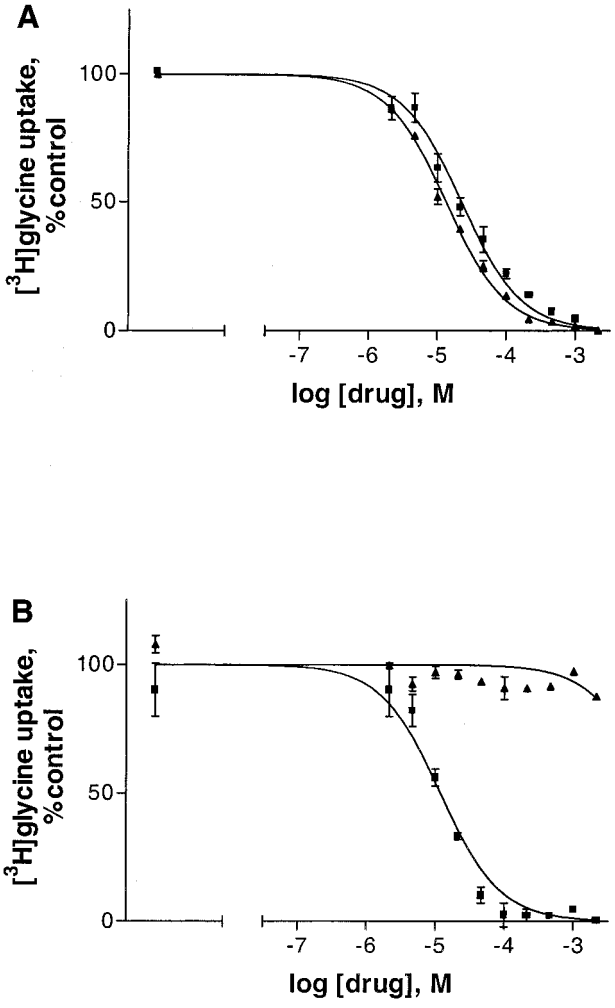


Fig. 2. Glycine transport in rat brain and spinal cord aggregates. Rat brain (A) or spinal cord (B) aggregates were prepared as described under *Materials and Methods* and incubated with the indicated concentrations of unlabeled glycine or sarcosine. [^{3}H]Glycine was added, and uptake was determined as described. Results are the means ± S.E.M. of three determinations expressed as percent of uptake in absence of inhibition. ■, glycine; ▲, sarcosine.

pcDNA3. Each of the glycine transport genes was subsequently expressed in QT6 cells, a quail fibroblast line suitable for stable expression of transporters (Borden et al., 1999), as described under *Materials and Methods*. The cell lines expressing the human GlyT1C and GlyT2 subtypes were selected for more detailed study. Transport of [^{3}H]glycine in the cell line stably transfected with the GlyT1C isoform was saturable (Fig. 4), sodium- and chloride-dependent (Fig. 5), and exhibited a K_m value of approximately 60 μM, which is consistent with characteristics described previously for these transporters (Kim et al., 1994). Specific transport was typically 90% of total radioactivity. We determined that the QT6 cell lines that were stably transfected with the GLYT transporter subtypes would be appropriate models for screening compounds that would discriminate between subtypes.

Pharmacology of Glycine Transport in QT6-1C Cells. [^{3}H]glycine transport in the QT6 cell line expressing the GlyT1C isoform was inhibited completely by unlabeled glycine or sarcosine. The IC_{50} value of sarcosine for inhibition of glycine transport in the QT6-1C cells was 9 μM, which is nearly identical to the IC_{50} value of 13 μM demonstrated in rat brain aggregates (Fig. 6). ALX 5407 inhibited [^{3}H]glycine transport in the QT6-1C cells completely, with an IC_{50} value of 3 nM, but had no effect on [^{3}H]glycine transport in QT6 cells stably transfected with the GlyT2 gene (Fig. 7).

ALX 5407 Exhibits No Activity at Other Glycine Binding Sites or Transporters. To establish that ALX 5407 is working selectively at hGlyT1, we measured the

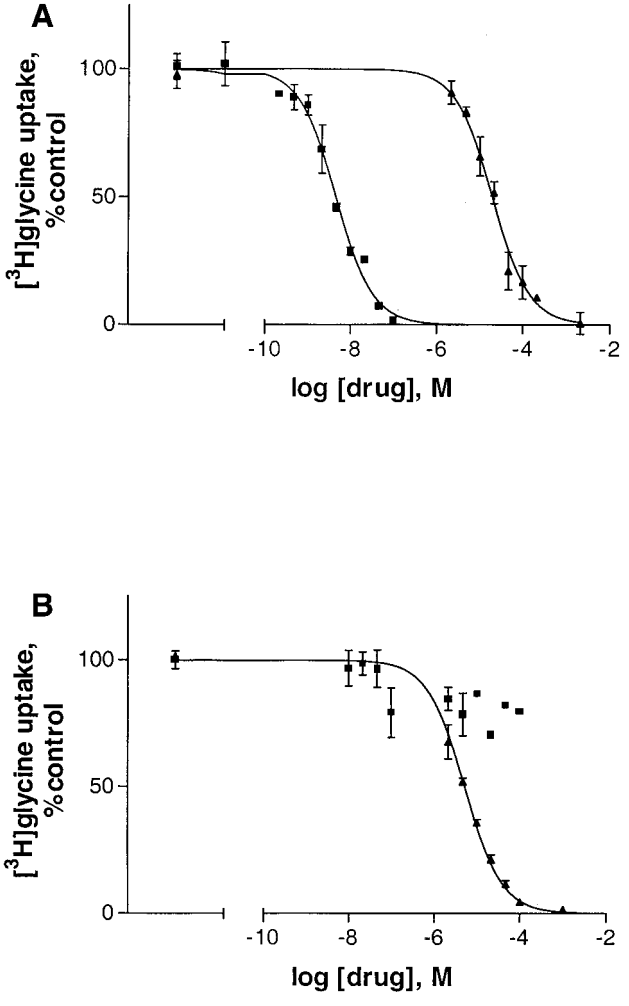


Fig. 3. ALX 5407 blocks [^{3}H]glycine transport in rat brain but not spinal cord aggregates. Rat brain (A) or spinal cord (B) aggregates were prepared as described under *Materials and Methods* and incubated with the indicated concentrations of ALX 5407. [^{3}H]Glycine was added, and uptake was determined as described. Results are the means ± S.E.M. of three determinations expressed as percentage of uptake in absence of inhibition. ■, ALX 5407; ▲, glycine.

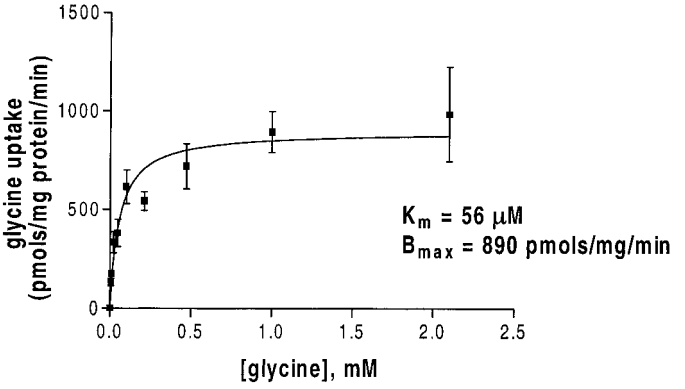
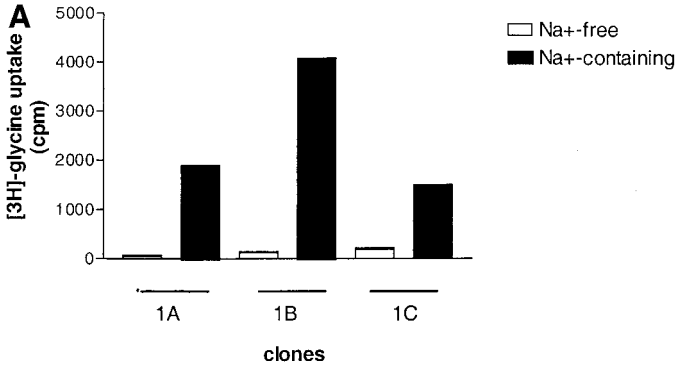


Fig. 4. Saturation curve for glycine transport in QT6 cells transfected with hGlyT1C. [^{3}H]Glycine uptake was measured in the presence of increasing concentrations of unlabeled glycine. Data are expressed as picomoles of glycine uptake per milligram of protein per minute. Measurements were repeated several times with similar results.



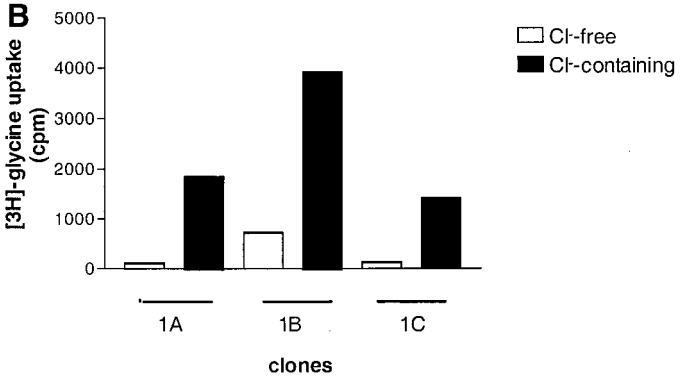


Fig. 5. [^{3}H]Glycine uptake in QT6-1C cells is Na^+ and Cl^- dependent. [^{3}H]glycine uptake was determined in QT6 cells expressing the hGlyT1A, -B, or -C variants in the presence or absence of sodium (A) or chloride (B) in the HBS assay buffer. Sodium in the HBS was substituted on a molar basis with choline, chloride with acetate. Data are from a single experiment that was repeated three times with similar results.

affinity of ALX 5407 at two other glycine recognition sites, the binding site on the NMDA receptor (strychnine-insensitive site) and the inhibitory glycine receptor (strychnine-sensitive site). Specific binding of [^{3}H]MDL 105,519 was approximately 70% of total binding and was not decreased in the presence of 100 μM ALX 5407. Specific binding of [^{3}H]strychnine to rat spinal cord membranes was approximately 75% of total binding, as defined by either 10 mM glycine or 100 mM unlabeled strychnine, and was not decreased in the presence of 100 μM ALX 5407.

ALX 5407 Exhibits Slow Dissociation Kinetics in QT6-1C Cells. The rate at which ALX 5407 dissociates from the GlyT1C transporter was measured using QT6-1C cells. The cells were pretreated with 50 nM ALX 5407, then washed four times with HBS. After washing, 90 μl of HBS was added, and the cells assayed for [^{3}H]glycine transport at the times indicated (Fig. 8). The long half-time indicates the binding of ALX 5407 to the GlyT1C transporter is essentially irreversible.

ALX 5407 Increases Free Glycine Levels in Rat Prefrontal Cortex. We used in vivo microdialysis to examine what effect ALX 5407 would have on extracellular levels of glycine in rat prefrontal cortex (PFC). Administration of ALX 5407 (10 mg/kg, p.o.) resulted in a 40% increase in PFC glycine levels measured 60 to 90 min after drug administration (Fig. 9), whereas the lower dose (1 mg/kg) elicited a slight, nonsignificant elevation.

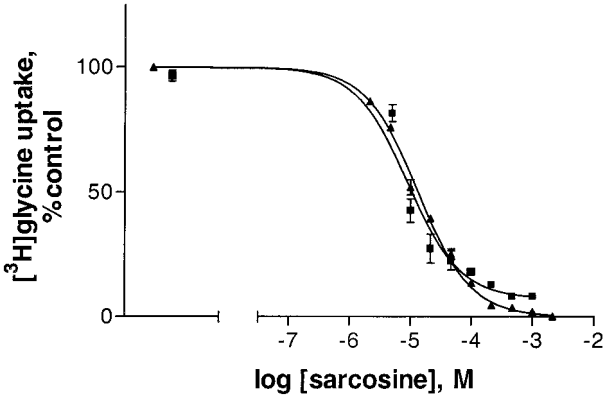


Fig. 6. Sarcosine inhibits [^{3}H]glycine uptake in rat brain aggregates or QT6-1C cells with similar potency. [^{3}H]glycine uptake was determined in rat brain aggregates or QT6-1C cells in the presence or absence of sarcosine. Data are triplicate determinations from a single experiment expressed as percentage of glycine uptake in the absence of inhibition. ■, QT6-1C; ▲, aggregates.

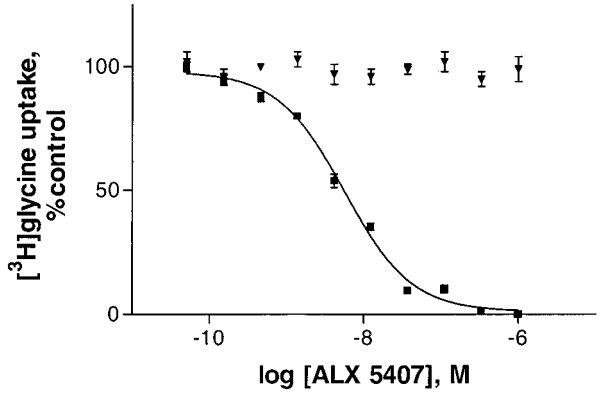


Fig. 7. ALX 5407 selectively inhibits the hGlyT1 glycine transporter. [^{3}H]glycine uptake was determined in QT6 cells expressing the hGlyT1 or hGlyT2 transporters in the presence or absence of ALX 5407. Data are triplicate determinations from a single experiment expressed as percentage of glycine uptake in the absence of inhibition. ■, hGLYT1C; ▼, hGLYT2.

Discussion

To enhance NMDA receptor function, we adopted the strategy of blocking glycine reuptake. Because the distribution of GlyT1 in rat more closely resembles the distribution of NMDA receptors than does GlyT2, we targeted GlyT1 as a potential mediator of NMDA function. Sarcosine, the *N*-methylated derivative of glycine, selectively inhibits GlyT1 compared with GlyT2. We have synthesized analogs of sarcosine in an attempt to make potent and selective inhibitors of GlyT1 (glycine reuptake inhibitors). Our hypothesis is that blocking GlyT1 in the CNS would raise glycine levels in vivo so that NMDA function would be augmented. A GlyT1 antagonist would serve this purpose.

The current study clearly indicates that ALX 5407 is potent and selective for the GlyT1 family of glycine transporters over GlyT2. ALX 5407 demonstrates these characteristics in both aggregate preparations of brain and spinal cord tissues (Fig. 3) or in a cell line stably transfected with either the GlyT1c or GlyT2 transporter (Fig. 7). The splice variants of the GlyT1 transporter are localized to different cell types (Borowsky et al., 1993). ALX 5407 does not seem to distinguish between the known variants of the GlyT1 transporter (data not shown). It is unclear what the implications of blocking all GlyT1 variants might be versus antagonizing a selected variant. Given the degree of homology in the amino acid sequence between the known variants, it may prove difficult to pharmacologically distinguish them.

Blocking the reuptake of glycine will potentiate the effects of the NMDA receptor only if the NMDA receptor is not saturated with glycine under physiological conditions. It is clear that blocking the glycine site either in an in vitro slice or in vivo will result in an inhibition of NMDA function (Johnson and Ascher, 1987; Thomson et al., 1989; Watenabe et al., 1992), demonstrating that glycine is required for NMDA receptor activation. D-Cycloserine, an NMDA receptor glycine site agonist, potentiates CNS-mediated NMDA responses, strengthening the argument that glycine receptors

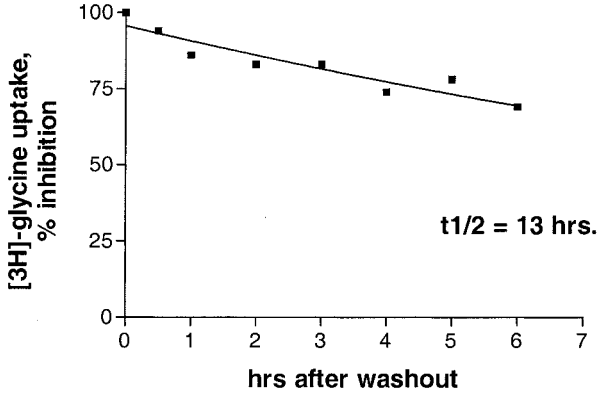


Fig. 8. The inhibitory effect of ALX 5407 on the hGlyT1C transporter has a half-time of approximately 13 h. [^{3}H]Glycine uptake was measured in QT6-1C cells in the presence or absence of 60 nM ALX 5407. Uptake was measured before and after wells were washed with HBS at times indicated. Data are triplicate determinations from a single experiment expressed as percentage of glycine uptake in the absence of inhibition.

are not saturated in vivo. Ambient concentrations of glycine are in the low micromolar range in cerebrospinal fluid (Kemp and Leeson, 1993), which would be high enough to saturate most NMDA receptors. Synaptic glycine concentration may be substantially lower than that of the cerebrospinal fluid if sufficiently regulated by the presence of glycine transporters. Recent evidence from studies using ALX 5407 in a hippocampal slice preparation indicates that this may be the case (Bergeron et al., 1998). The current study reinforces the idea that a GlyT1 antagonist can substantially alter local glycine concentrations. The in vivo microdialysis experiments indicate that ALX 5407 can increase glycine concentrations in rat PFC by at least 40% at a dose that may represent occupancy of only 50% of the available GlyT1 sites (Fig. 9). This increase in glycine is probably even higher in the synaptic cleft, because the microdialysis measurements may simply reflect extrasynaptic glycine levels. However, glycine was the only amino acid measured in this study, and additional studies should be undertaken to confirm that the observed results are a direct effect of ALX 5407 on glycine uptake and not a nonspecific effect on amino acids. Interestingly, there were no signs of cytotoxicity or seizures during administration of ALX 5407. In addition to its extracellular role as a neurotransmitter, glycine serves a number of important metabolic roles intracellularly. A glycine transport blocker that was itself transported would be undesirable because it would accumulate within the cell and, given its similarity to glycine, potentially interfere in metabolic routes that involve glycine. ALX 5407 is not transported into cells (data not shown) and therefore should not accumulate intracellularly. ALX 5407 does not recognize other glycine sites, including the glycine site on the NMDA receptor or the strychnine-sensitive receptor. If it did, the effects at the transporter would be mitigated, because in addition to raising glycine levels, it would also prevent glycine from activating NMDA receptors.

Deficits in glutamatergic transmission are linked to cognitive deficits and schizophrenia. In particular, there are data suggesting a link between schizophrenia and the *N*-methyl-D-aspartate receptor. Agents that block the NMDA channel (PCP and ketamine) produce a psychotic-like state that closely resembles schizophrenia in man and enhance psychotic episodes in schizophrenics. Furthermore, positron emission tomography images show that schizophrenics demonstrate aberrant patterns of energy metabolism in those parts of the brain that are thought to be primarily affected by schizophrenia (Tamminga, 1998). There is also evidence for reduced NMDA-mediated glutamate release in preparations of schizophrenic brain (Sherman et al., 1991). Administration of agents that act as agonists at the NMDA-glycine site (glycine, D-cycloserine, D-serine) have demonstrated efficacy in clinical trials. Various hypotheses have been put forth to explain the link in terms of neurological circuits. One hypothesis states that glutamatergic inputs are linked to cortical cholinergic neurons by an inhibitory γ-aminobutyric acid pathway; loss of the glutamatergic input disinhibits the cholinergic neurons, and this activity accounts for psychotic episodes. Another hypothesis states that the balance of glutamatergic and dopaminergic input to an intracellular signal in striatal neurons is necessary to establish the correct feedback between basal ganglia and cortical neurons; schizophrenia would result from too much dopaminergic or too little glutamatergic transmission (Carlson and Carlson, 1990). Whatever the mechanism, pharmacological potentiation of NMDA receptor output would be desirable to test the hypothesis that hypofunction of glutamatergic transmission underlies some forms of schizophrenia.

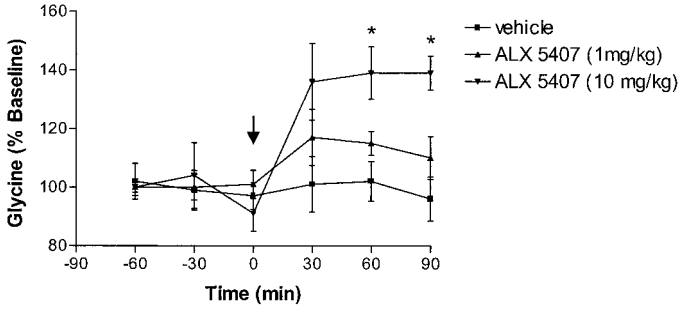


Fig. 9. Effects of ALX 5407 on extracellular glycine levels in rat prefrontal cortex. Responses to p.o. administration of vehicle (30% PG, $n = 5$) or ALX 5407 (1 mg/kg, $n = 5$ or 10 mg/kg, $n = 3$). Data are presented as the mean ± S.E.M. normalized to percent change from baseline. Basal levels of glycine are taken as the mean of the three samples just before vehicle or drug administration (noted by arrow). Mean basal levels of glycine were 6.5 ± 1.9 pmol/5 μl. *, time points that are significantly different between vehicle and ALX 5407 (10 mg/kg; $p < 0.05$).

Agonists acting at the glycine site of the NMDA receptor may potentially be useful for improving cognition or learning. In vitro slice data demonstrates that glycine-site agonists like D-serine and D-cycloserine potentiate long-term potentiation. Conversely, long-term potentiation can be inhibited by drugs acting as antagonists at the NMDA/glycine site (Bashir et al., 1991). 7-Chlorokyneurinic acid, an NMDA/glycine site antagonist, produces learning impairment in rats studied in passive avoidance paradigms and inhibits spatial learning in a Morris water maze paradigm (Watenabe et al., 1992).

The results of the current study indicate ALX 5407 is a potent, selective inhibitor of the GlyT1 family of glycine transporters. In vivo microdialysis suggests that ALX 5407 binds to GlyT1 transporters in the CNS after oral dosing, and that the pharmacological activity of ALX 5407 results in a measurable physiological response.

It remains to be seen whether this new class of pharmacological agents will demonstrate sufficient safety and efficacy to make them useful in the treatment of schizophrenia or other cognitive disorders. They should prove to be an additional tool in the characterization of the role glycine and NMDA receptors play in CNS neurotransmission.

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