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Inhibitors of GlyT1 affect glycine transport via discrete binding sites

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Running Title: Competitive and non-competitive GlyT1 inhibitors

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Discussion: 1221

Abbreviations: NMDAR: N-methyl-D-aspartate receptor; GlyT1: glycine transporter subtype 1; (R)-NPTS: (R)-N[3-phenyl-3-(4´-(4-toluoyl)phenoxy)-propyl]sarcosine, NFPS/ALX 5407: (R)-N[3-(4´fluorophenyl)-3-(4´phenyl-phenoxy)propyl]-sarcosine, SSR504734: 2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide, monohydrochloride, N-methyl-SSR504734: 2-chloro-N-[(S)-phenyl[(2S)-N-methylpiperidin-2-yl]-methyl]-3-trifluoromethyl benzamide monohydrochloride; Org24598: R,S-(+/-)N-methyl-N-[(4-trifluoromethyl) phenoxy]-3-phenyl-propylglycine

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Abstract

In the forebrain, synaptic glycine concentrations are regulated through the glycine transporter GlyT1. Since glycine is a co-agonist of the NMDA receptor (NMDAR), which has been implicated in schizophrenia, inhibition of GlyT1 is thought to provide an option for the treatment of schizophrenia. In support of this hypothesis, GlyT1 inhibitors facilitate *in vivo* NMDAR function and demonstrate antipsychotic-like effects in animal models. Among the specific GlyT1 inhibitors, substituted N-methyl-glycine (sarcosine) derivatives (e.g. NFPS, (R)-NPTS and Org24589), and non-sarcosine containing inhibitors, such as SSR504734, have been described. In the present study, we analyzed the mode of interaction of these compounds with GlyT1 by using electrophysiological measurements in *Xenopus* oocytes, and with two binding assays, using [^{3}H]-(R)-NPTS or [^{3}H]-N-methyl-SSR504734 as radioligands. Inhibition of electrogenic glycine transport by sarcosine-based compounds was apparently irreversible and independent of glycine concentration. The latter indicates a non-competitive mode of action. In contrast, both SSR504734 and N-methyl-SSR504734 exhibited reversible and competitive inhibition of glycine transport. In GlyT1-expressing membranes, the binding of the novel radioligand [^{3}H]-N-methyl-SSR504734 to a single site on GlyT1 was competitively displaced by glycine and SSR504734, but non-competitively by sarcosine-based compounds. Conversely, [^{3}H]-(R)-NPTS binding was competitively inhibited by sarcosine-based compounds, while glycine, SSR504734 and N-methyl-SSR504734 non-competitively decreased maximal binding. Our data indicate that besides exerting an apparently irreversible or reversible inhibition, GlyT1 inhibitors differ by exhibiting either a non-competitive or competitive mode of inhibition. The divergent modes of inhibition may significantly impact the efficacy and tolerability of these drugs.

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Introduction

Glycine acts as a neurotransmitter at inhibitory glycine receptors (glycine A receptors) as well as an essential co-agonist of the N-methyl-D-aspartate (NMDA)-type glutamate receptor. The extracellular glycine concentration in the brain is regulated by the high affinity glycine transporters, GlyT1 and GlyT2 (Betz et al., 2006).

GlyT1 and GlyT2 possess different expression patterns, pharmacology and transport stoichiometries. Because of its stoichiometry of 2 Na^+/1 Cl^-/1 glycine, GlyT1 is ideally suited to transport glycine into and out of the cell throughout a large concentration range and at different membrane potentials, thereby controlling synaptic glycine concentrations (Roux and Supplisson, 2000). Besides its expression in caudal regions of the CNS, GlyT1 is also present in the forebrain. There, it is localized in astrocytes as well as in pre- and postsynaptic terminals of glutamatergic synapses, where GlyT1 was shown to be physically associated with PSD95, an NMDAR associated protein (Cubelos et al., 2005). These findings support a crucial role of GlyT1 in controlling NMDAR activity by regulating synaptic glycine concentrations.

Hypofunction of NMDAR is thought to be implicated in schizophrenia (Coyle et al., 2006; Millan, 2005). The most compelling evidence for a pathophysiological role of impaired NMDAR originates from the discovery of several schizophrenia susceptibility genes linked to NMDAR (Harrison and Weinberger, 2005; Morrison and Pilowski, 2007). Another line of evidence is provided by the behavioral and neurophysiological effects of NMDAR antagonists, which resemble key aspects of schizophrenia (Javitt and Zukin, 1991; Lahti et al., 1995; Morris et al., 2005). Enhancing NMDAR activity by increasing glycine levels via GlyT1 inhibition has been

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demonstrated (Bergeron et al., 1998) and is being pursued as an approach for the treatment of schizophrenia (for reviews see Lechner, 2006; Lindsley et al., 2006).

GlyT1 inhibitors belong to diverse structural classes (Lechner, 2006; Lindsley et al., 2006). Like sarcosine, substituted sarcosine derivatives, such as (R)-NPTS, NFPS (ALX5407), and Org24598, inhibit GlyT1, but not GlyT2. Of these, NFPS and Org24598 have been used to evaluate the concept of facilitating NMDAR signaling by GlyT1 inhibition. The compounds were shown to enhance extracellular glycine levels (Atkinson et al., 2001), to facilitate NMDAR activity (Chen et al., 2003), and to enhance long term potentiation (LTP) in the rat hippocampus *in vivo* (Kinney et al., 2003). Further *in vivo* studies revealed an antipsychotic-like profile in animal models, including inhibition of PCP-induced hyperlocomotion (Harsing et al., 2003), reversal of impaired prepulse inhibition (PPI), and a pattern of c-fos expression resembling that of clozapine (Kinney et al., 2003). While showing promising properties in disease-related models, sarcosine-based compounds have also been reported to exert toxic effects (Harsing et al., 2006) by an unknown mechanism. Potentially, this may be due to apparent irreversible GlyT1 inhibition, which has been reported for NFPS (Aubrey and Vandenberg, 2001). Recently, a non-sarcosine, reversible GlyT1 inhibitor (SSR504734) has been described (Depoortère et al., 2005). The compound was shown to elevate brain glycine levels, enhance glutamatergic neurotransmission, and to be effective in several models predictive of antipsychotic activities (Depoortère et al., 2005). In spite of this extensive characterization, a detailed analysis of the molecular mode of action at GlyT1 was not released.

Here we compared the mechanism of interaction of two non-sarcosine-based GlyT1 inhibitors (SSR504734 and N-methyl-SSR504734) with those of three sarcosine-based compounds (NFPS, (R)-NPTS and Org24589). In particular, we

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investigated whether the two classes of compounds affect the same or different binding sites at the transporter, and if the interaction site is orthosteric or allosteric to the functional glycine site.

To this end, we studied the inhibitory mechanisms of the compounds by measuring the electrogenic glycine transport in GlyT1-expressing *Xenopus* oocytes. Furthermore, we developed a new binding assay based on [^{3}H]-N-methyl-SSR504734 and employed the recently described binding assay using [^{3}H]-(R)-NPTS (Lowe et al., 2003). We outline that these structural classes of GlyT1 inhibitors act at different binding sites on GlyT1. The implications of these findings for the pharmacological properties of GlyT1 inhibitors will be discussed.

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Materials and Methods

Materials

(R)-N-[3-phenyl-3-(4´-(4-toluoyl)phenoxy)-propyl]-sarcosine ((R)-NPTS) was synthesized at Abbott according to described procedures in patent number WO2002000602. (R)-N[3-(4´fluorophenyl)-3-(4´phenyl-phenoxy)propyl]-sarcosine (ALX 5407, NFPS) and R,S-(+/-)N-methyl-N-[(4-trifluoromethyl) phenoxy]-3-phenyl-propylglycine (Org24598) were commercially available, and 2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide, monohydrochloride (SSR504734) was synthesized at Abbott according to described procedures in patent number WO2003089411A1.

For 2-chloro-N-[(S)-phenyl[(2S)-N-methylpiperidin-2-yl]-methyl]-3-trifluoromethyl benzamide mono-hydrochloride (N-methyl-SSR504734), which is a methyl-derivative of SSR504734, the tritium label was introduced as follows: A solution of 2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl]-methyl]-3-trifluoromethyl benzamide mono-hydrochloride (SSR504734, 4 mg, 0.01 mmol) in tetrahydrofuran (2 ml) was charged to a 5-ml round bottom flask equipped with a magnetic stirrer bar. To this solution, aqueous formaldehyde (50 µl) and triethylamine (0.1 ml) were added followed by Pd/C (10%, 8 mg) and this mixture was tritiated (tritium, 1.9 Ci) in a trisorber for 15 h. The excess tritium was pushed back in a recovery bed and the reaction flask was purged with helium. The reaction mixture was diluted with methanol and the catalyst was filtered. The labile tritium was removed by treating with methanol (3 x 5 ml) and concentrated. The residue was taken in methanol (10 ml) and radioactivity was measured as 89 mCi. The solvent was concentrated and the residue was purified by preparative HPLC. The crude product was evaporated to dryness and the residue was dissolved in 1.2 ml of acetonitrile with 0.1% TFA. Purification was accomplished

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by HPLC on an Agilent 1100 series HPLC system with ChemStation software. Approximately 450 µl of this sample was injected onto a Phenomenex Luna C 18 column (5 microns, 250 mm x 10 mm ID) with a gradient mobile phase of 2% to 95% B for 35 min (Mobile phase A = 0.1% TFA/water and Mobile Phase B = 0.1% TFA/acetonitrile) at a flow rate of 4 ml/min with an UV detection at 254 nm. The fractions containing [^{3}H]-N-methyl-SSR504734 were collected at approximately 18 min using an Agilent fraction collector. The product containing fractions were pooled and solvents were removed by evaporation in vacuo. The residue (9 mCi, 99% pure) was dissolved in 4 ml of 200-proof ethanol. The specific activity was determined to be 11.4 Ci/mmol from the ratio of the mass spectroscopic measurement of the isotopic molecular ions. Tritium labeling of (R)-NPTS ([^{3}H]-(R)-NPTS) was carried out by Biotrend Chemikalien GmbH (Köln, Germany).

Expression of hGlyT1 in mammalian cells

HEK293 and CHO cells were stably transfected with a pcDNA3 expression plasmid encoding the full length human GlyT1c transporter. Cell clones hGlyT1c_5_CHO and hGlyT1c_48_HEK293 resistant to G418 were selected for binding and functional uptake studies. Human splice variants GlyT1a, GlyT1b and GlyT1c (Kim et al., 1994) in pcDNA3 expression plasmid were transiently expressed in FreeStyle HEK293 (HEK293-F) cells (Invitrogen).

Functional expression in *Xenopus* oocytes

Female *Xenopus laevis* (Nasco, USA), were anaesthetized in solution with 0.2% Tricain (Sigma) and 2 g/l sodiumhydrogencarbonate (Sigma), ovary lobes were removed, and oocytes were released from the follicle tissue with collagenase (Type I, 2 mg/ml for 2hr, Roche Applied Science, Mannheim, Germany). Stage V and VI oocytes were selected by hand and each oocyte was injected with 20 nl of cDNA

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solution (10 ng/ml in water) into the nucleus. Alternatively, cRNA was transcribed with T7 RNA polymerase from pGemHeJuel plasmids (Liman et al., 1992) containing either human GlyT1c or human GlyT2a and capped with 5'-7-methyl guanosine using the mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX, U.S.A.). Oocytes were injected with 50 nl (300 ng/ml) RNA solution. The cells were then incubated for 2-5 days at 18 °C in Barth medium (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 5 mM Tris-HCl, pH 7.4) supplemented with gentamicin (50 mg/ml, Sigma). Oocytes were used for membrane preparations and electrophysiological uptake measurements.

Membrane preparation from GlyT1c-expressing *Xenopus* oocytes and recombinant CHO and HEK293 cells

GlyT1c expressing cells were pelleted and washed twice in cold PBS, containing 2 mM EDTA and snap frozen in liquid nitrogen or ethanol/dry ice. The thawed cell pellets were resuspended in ice-cold sucrose buffer (0.25 M sucrose, 10 mM HEPES, 1 mM PMSF, 5 µg/ml pepstatin A, 3 mM EDTA, 0.025% bacitracin, pH 7.4) and homogenized in 5 ml aliquots by sonication in a tissue homogenizer. Cell breakage was monitored by light microscopy. The homogenate was centrifuged for 10 minutes at 1,000 x g at 4°C to pellet remaining unbroken cells. The sucrose buffer supernatant was centrifuged at 60,000 x g at 4°C for 1 hr. The resulting pellet was resuspended in 20 ml of cold 20 mM Tris-HCl, pH 7.4, containing 5 µg/ml pepstatin A, 0.1 mM PMSF, 3 mM EDTA and centrifuged again at 60,000 x g at 4°C for 1 hr. After a final resuspension of the pellet by help of sonication, the protein concentration was determined and the suspension was snap frozen in aliquots and stored at -80°C.

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[^{3}H]-(R)-NPTS or [^{3}H]-N-methyl-SSR504734 radioligand binding assays

Radioligand binding to human GlyT1c transporter-expressing membranes was measured in duplicate in a total volume of 200 µl in 96-well plates. To 100 µl of membrane suspension in assay buffer (120 mM NaCl, 2 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5), 80 µl of [^{3}H]-(R)-NPTS (0.5 nM final) or [^{3}H]-N-methyl-SSR504734 were added in assay buffer, yielding a final membrane protein concentration of 50 µg/ml. In competition experiments, 10 µl of buffer or unlabeled compound solution were added. The final DMSO concentration was 1% in all cases. Non-specific binding was determined in the presence of 10 µM Org24598 (or its racemate Org24461) for [^{3}H]-(R)-NPTS or 10 µM SSR504734 for [^{3}H]-N-methyl-SSR504734. After incubation at room temperature for 1h, the incubation mixture was harvested (Tomtec Mach III U Harvester) through 96-well GF/B filter plates (PerkinElmer), presoaked for 1hr with 40 µl per well of 0.1% polyethyleneimine. After washing twice with ice-cold buffer (50 mM Tris-HCl, pH 7.4), plates were dried and 35 µl scintillator (BetaplateScint, PerkinElmer) were added per well. The radioactivity was determined by liquid scintillation spectrometry in a MicroBeta (PerkinElmer) plate counter.

Data analysis: For binding of [^{3}H]-(R)-NPTS or [^{3}H]-N-methyl-SSR504734 to cell membranes, the calculation of K_d and B_{max} values from the saturation binding assays and of the IC_{50} values from the displacement binding was performed by iterative non-linear regression analysis adapted from the 'Ligand' program (Munson und Rodbard, 1980). Radioligand saturation binding and displacement curves in the presence or the absence of tested compounds were fitted using a one-site or a two-site fit, and the more suitable model was identified by a partial F-test (De Lean et al., 1982).

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Apparent K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Glycine uptake in recombinant hGlyT1 expressing cells

Human GlyT1c expressing recombinant hGlyT1c_5_CHO or hGlyT1c_48-HEK293 cells or FreeStyle HEK293-F cells transiently transfected with human GlyT1a, GlyT1b or GlyT1c were plated at 20,000 cells per well in 96 well Cytostar-T scintillation microplates (Amersham Biosciences) and cultured to sub-confluency for 24 hr. For glycine uptake assays the culture medium was aspirated and the cells were washed once with 100 µl HBSS (Gibco BRL, #14025-050) containing 5 mM L-alanine (Merck #1007). 80 µl HBSS buffer was added, followed by 10 µl inhibitor or vehicle (10% DMSO) and 10 µl [^{3}H]-glycine (TRK71, Amersham Biosciences). The final glycine concentration was 250 nM (if not stated otherwise). The plates were placed in a Wallac Microbeta (PerkinElmer) and continuously counted by solid phase scintillation spectrometry up to 3 hr. Nonspecific uptake was determined in the presence of 10 mM unlabeled glycine or 10 µM of Org24598 (IC_{50} calculations were made by four-parametric logistic nonlinear regression analysis (GraphPad Prism)), using determinations within the range of linear increase of [^{3}H]-glycine incorporation.

Glycine uptake in mouse astrocytes

Experimental procedures were approved by Abbott's Animal Welfare Office and were performed in accordance with the German national guidelines as well as recommendations and policies of the U.S. National Institutes of Health "Principles of laboratory animal care" (1996 edition). Animal housing and experiments were conducted in the facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Primary mouse astrocytes were prepared from C57BL6 mice (Janvier) and cultured in DMEM with

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10% FCS and 1% PenStrep under 10% CO_2. 24 hr prior to the experiment, the medium was substituted by serum-free Neurobasal/B27 medium and cells were plated at 15,000 cells per well in 96 well Cytostar-T scintillation microplates (Amersham Biosciences). After washing the cells once with 100 µl HBSS (Gibco BRL, #14025-050), containing 5 mM L-alanine (Merck, #1007), the glycine uptake assay was performed as described above.

Electrophysiological measurements in *Xenopus* oocytes

The measurement of the membrane current on whole oocytes was carried out as described (Methfessel et al., 1986; Mezler et al., 2001). The cells were penetrated with two microelectrodes with a resistance < 1.5 MΩ, filled with 1.5 M K-acetate plus 0.1 M KCl) in an acrylic plastic chamber. The membrane potential was held at -60 mV with a voltage clamp amplifier (TEC 03X, npi, Tamm, Germany), and the membrane current was recorded at 20 Hz. The measurement chamber was continuously perfused with frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2). All compounds were diluted in frog Ringer solution, measurements were at room temperature. To administer compounds, the software-driven perfusion was switched between reservoirs with the appropriate solutions. The flow rate of 5 ml/min resulted in an exchange of solutions at the oocyte within 1-2 sec. Data acquisition and analysis were performed with CellWorks 5.5 software (npi, Tamm, Germany) and GraphPad Prism.

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Results

In order to study differences in their interaction kinetics, GlyT1 inhibitors from two different structural classes were investigated; the first class comprising SSR504734 and N-methyl-SSR504734, the second class comprising the N-methyl-glycine (sarcosine) derivatives (sarcosine-based compounds) NFPS, (R)-NPTS and Org24598 (Fig. 1).

All tested compounds are potent and selective GlyT1 inhibitors without splice-variant selectivity

Steady state inhibition experiments of [^{3}H]-glycine uptake in mouse astrocytes and CHO cells recombinantly expressing human GlyT1c demonstrated that all tested GlyT1 inhibitors completely blocked [^{3}H]-glycine uptake with nanomolar potency. In mouse astrocytes the rank order was NFPS>(R)-NPTS>N-methyl-SSR504734>Org24598>>SSR504734 (Table 1, first column). The rank order of potency was similar in the recombinant system, expressing the human GlyT1c transporter (Table 1, middle column), but generally, the potency of inhibition was reduced. All compounds tested, as well as the substrates glycine and sarcosine, displaced binding of the new radioligand [^{3}H]-N-methyl-SSR504734 (Table 1, right column). To rule out substantial differences in the inhibitory potency of the compounds for the GlyT1 subtypes, we tested the inhibition of glycine uptake for cells expressing either hGlyT1a, hGlyT1b or hGlyT1c. Org24598 and SSR504734 were used as representatives for sarcosine-based and non-sarcosine-based inhibitors, and were tested for inhibition of [^{3}H]-glycine uptake in GlyT1-expressing HEK293-F cells, transiently transfected with the three human GlyT1 splice variants. The IC_{50} values for the different subtypes were not significantly different ($p>0.05$ by Kruskal-Wallis test; data not shown). Based on these and previously published results, the subsequent

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experiments were carried out with hGlyT1c only. None of the compounds demonstrated inhibition of GlyT2-mediated glycine transport in radioactive uptake experiments in recombinant GlyT2-expressing cells or electrophysiological measurements in *Xenopus* ooyctes (data not shown).

SSR504734 and N-methyl-SSR504734 are potent, reversible and orthosteric inhibitors of electrogenic GlyT1 transport in *Xenopus* oocytes

Previously, *Xenopus* oocytes were described as a useful system to functionally express and characterize GlyT1 (Roux and Suplisson, 2000; Aubrey and Vandenberg, 2001). To evaluate the inhibitory properties of SSR504734, we first stimulated the glycine transport through human GlyT1c by adding glycine (20 µM) at a concentration corresponding approximately to its K_M, followed by a co-application of 20 µM glycine and 3 µM SSR504734, and a washout period, in which 20 µM glycine was applied in the absence of SSR504734 (Fig. 2A). SSR504734 proved to be an effective and reversible inhibitor of electrogenic glycine transport also in the *Xenopus* oocyte system. The compound blocked the transport of 20 µM glycine with an on-rate ($t_{1/2\ on}$) of 11 ± 1 sec (n=3) and the inhibition of glycine transport was readily reversible with an off-rate ($t_{1/2\ off}$) of 40.2 ± 8.3 sec (n=3).

If the inhibitor binding site is orthosteric to the site bound by glycine, it should be possible to displace the inhibitor with increasing glycine concentrations, and the high substrate concentration should protect the transporter from inhibition (surmountability). Non-surmountable compounds would preclude the agonist to attain the maximal signal. Therefore, we evaluated the inhibition of the compound by co-application of a defined concentration of the inhibitor together with a low (10 µM; about ½ K_M) and a high (3 mM, about 150x K_M) concentration of glycine. In our surmountability protocol, after a control stimulus with 20 µM glycine, hGlyT1c was

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activated by glycine (10 µM or 3 mM), followed by a co-application of 3 µM SSR504734 together with 10 µM and 3 mM glycine respectively (Fig. 2B, C, D). While at 10 µM glycine the current is blocked by 82 ± 2% (n=5), the inhibition is significantly ($p<0.0001$; t-test) reduced with 3 mM glycine. Only a residual inhibition of 36 ± 3% (n=6) is left under these conditions (Fig.2B).

To further validate a potential competitive mode-of-action for SSR504734, we tested the glycine-dependence of the inhibition in more detail. Because SSR504734 inhibition is quickly reversible, we were able to test the compound at concentrations of 0, 1, 3 and 10 µM together with a glycine concentration response curve. The results for 0 µM and 10 µM are shown (Fig. 3A, B upper panel). Without inhibitor the EC_{50} of glycine is 19.4 ± 5.7 µM (Fig. 3A; n=7). At higher inhibitor concentrations, the glycine EC_{50} is shifted to larger values. For example, at 10 µM SSR504734, the EC_{50} of glycine is 592 ± 40 µM (Fig. 3B; n=4). According to a modified Gaddum/Schild model (Lazareno and Birdsall, 1993), a non-linear regression analysis of glycine concentration-response curves with increasing concentrations of SSR504734 resulted in a calculated Schild slope of 1 when subjected to global fitting (Fig. 3C, upper panel). These data suggest that inhibition of SSR504734 is competitive to glycine. Consequently, transformation into a linear Schild Plot resulted in a slope of 0.91 (Fig. 3C, lower panel). The K_b of SSR504734 determined from global non-linear fitting was 214 nM (Fig. 3C, upper panel). This value corresponds well to the IC_{50} value of this compound in the radioactive uptake assay in hGlyT1c-expressing CHO cells (see Table 1, middle column).

To facilitate the generation of a radioligand for GlyT1, we synthesized N-methyl-SSR504734. In *Xenopus* oocytes, similar to SSR504734, 3 µM N-methyl-SSR504734 blocked the glycine-induced current through GlyT1c (Fig. 4) with a $t_{1/2\ on}$ of 12 ± 0.3

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sec (n=4). Although the $t_{1/2\ off}$ with 868.4 ± 36 sec (n=4) was relatively slow, the inhibition clearly proved to be reversible. A representative trace of such an experiment is shown in Fig. 4A. Due to the slow off-rate of N-methyl-SSR504734, a direct Schild evaluation was not feasible. Therefore, the rate of inhibition was tested in separate experiments with two glycine concentrations as described above (surmountability). While 1 µM N-methyl-SSR504734 almost completely blocked the glycine current at 10 µM glycine (inhibition 91 ± 5 % (n=4); Fig. 4B; C), the inhibition was largely alleviated at 3 mM glycine (residual inhibition 26 ± 4 % (n=5); Fig. 4B; D). These findings are equivalent to the surmountability of SSR504734, for which we have validated the competitive inhibition with the Schild analysis.

Sarcosine-based compounds are apparently irreversible and non-competitive inhibitors of electrogenic GlyT1 transport in *Xenopus* oocytes

NFPS has previously been reported to be a non-reversible and non-surmountable inhibitor of GlyT1 transport (Aubrey and Vandenberg, 2001). Due to the structural similarity, we evaluated if these properties were also shared by other compounds of this class.

In a first set of experiments, we verified that all three sarcosine-based inhibitors are apparently irreversible inhibitors of GlyT1 in the *Xenopus* oocyte system (Fig. 5). At a concentration of 1 µM all three compounds blocked the transport of 20 µM glycine quickly ($t_{1/2\ on}$ of 23 ± 4 s (NFPS, n=4), 59 ± 4 s (NPTS, n=4) and 70 ± 2 s (Org24598, n=4)), and almost completely. The blockade was apparently irreversible, as an application of 20 µM glycine for 10 min did not reduce the inhibition of the compounds, and also after washout, 20 µM glycine did not induce an increased signal, but the current still is either completely absent (Fig. 5 A for NFPS) or

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corresponds to the residual current initially left by the inhibitor (Fig. 5 B; C for NPTS and Org24598).

Further, we evaluated whether the sarcosine-based inhibitors are competitive or non-competitive blockers of glycine transport. To avoid an influence of apparent irreversibility on the following glycine current, like before, the sarcosine-based inhibitors were tested in the surmountability protocol. 500 nM NFPS blocked the glycine current induced by 10 µM and 3 mM glycine to the same extent (91 ± 4 %; n=5; 90 ± 4 %; n=5; Fig. 6 A, B, C). This indicates that inhibition by NFPS is not surmountable by high glycine concentrations, and suggests a non-competitive inhibition of glycine transport. Likewise, 2 µM (R)-NPTS (Fig. 6D) and 1.5 µM Org24598 (Fig. 6E) inhibition was not surmountable. For (R)-NPTS, inhibition was 96 ± 3% at both glycine concentrations (10 µM: n=7 and 3 mM: n=4; Fig. 6D); for Org24598, the inhibition amounted to 93 ± 8 (n=8) and 96 ± 1 % (n=6) for 10 µM and 3 mM glycine, respectively (Fig. 6E).

Characterization of the new radioligand [^{3}H]-N-methyl-SSR504734 – saturation isotherms and binding kinetics

Our functional studies suggest that SSR504734 and N-methyl-SSR504734 are competitive inhibitors of glycine transport. Therefore, we assumed that SSR504734 and N-Methyl-SSR504734 bind to the glycine binding site of the transporter. For studying the interaction of inhibitors and glycine through binding studies, only tritiated (R)-NPTS and NFPS had been described as radioligands. In order to provide a radioligand for the proposed glycine binding site, we radiolabeled N-methyl-SSR504734. Saturation binding experiments with [^{3}H]-N-methyl-SSR504734 revealed a mean K_d of 8.1 nM for different preparations of *Xenopus* ooycte membranes, or 3.3 nM for hGlyT1c from CHO cell membranes. All inhibitors tested,

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as well as the substrates glycine and sarcosine, concentration-dependently displaced [^{3}H]-N-methyl-SSR504734 binding to human GlyT1c expressing CHO cell membranes (Table 1, right column).

Displacement of [^{3}H]-N-methyl-SSR504734 binding by sarcosine- and non-sarcosine containing compounds

More specifically, we measured [^{3}H]-N-methly-SSR504734 saturation-binding isotherms in absence and presence of increasing concentrations of the sarcosine-based inhibitors, SSR504734, and glycine, in recombinant CHO membranes expressing human GlyT1c, thereby testing for a competitive or non-competitive mode of displacement.

SSR504734, like glycine, significantly increased the K_d of the [^{3}H]-N-methly-SSR504734 binding while leaving the B_{max} unaffected (Fig. 7A, B, Table 2 upper panel). These data support a competitive interaction of these compounds at the [^{3}H]-N-methly-SSR504734 binding site, in support of the functional data.

In contrast, as a representative of the sarcosine-based compounds, (R)-NPTS decreased B_{max} and increased the K_d, indicating a mixed-model inhibition mechanism of [^{3}H]-N-methyl-SSR504734 displacement (Fig. 7C, Table 2 upper panel). Similar results were obtained for NFPS (data not shown).

Non-competitive displacement of [^{3}H]-(R)-NPTS binding by the glycine-competitive non-sarcosin containing N-methyl-SSR504734

For sarcosine-based GlyT1 inhibitors like (R)-NPTS the functional data in *Xenopus* oocytes, together with the displacement of [^{3}H]-N-methyl-SSR504734, suggest an allosteric binding site, distinct from the glycine binding site. [^{3}H]-(R)-NPTS was used as a radioligand to cross-check the binding of N-methyl-SSR504734, SSR504734 and glycine. Binding of [^{3}H]-(R)-NPTS to recombinant CHO membranes expressing

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human GlyT1c revealed a K_d value of 2.2 nM, when the data were fitted to a one- site model (data not shown).

As demonstrated in table 2 (lower panel) and Fig. 7 (D, F), increasing concentrations of SSR504734 and N-methyl-SSR504734 resulted in a significant decrease of the B_{max} of [^{3}H]-(R)-NPTS binding, while the K_d remained unchanged. With glycine (Fig. 7E) a similar trend was observed, although the B_{max} decrease did not reach statistical significance. Thus, the SSR compounds interact non-competitively with [^{3}H]-(R)-NPTS. Conversely, these findings are consistent with the competitive displacement of [^{3}H]-N-methyl-SSR504734 by SSR504734 and glycine. Similar data were obtained using *Xenopus* oocyte membranes (data not shown), indicating that the binding properties of the inhibitors to hGlyT1c are independent of the expression system. Further evidence for different binding sites of [^{3}H]-(R)-NPTS and [^{3}H]-N-methly-SSR504734 comes from a comparison of the potency to block radioligand binding. When generating the K_i ratio for [^{3}H]-N-methly-SSR504734 over [^{3}H]-(R)-NPTS binding displacement, the sarcosine-containing compounds display ratios above 1 (17 for ORG24598, 8 for (R)-NPTS and 3 for NFPS), whereas N-Methly-SSR504734 (0.15), SSR504734 (0.4) and glycine (0.75) display ratios below 1.

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Discussion

In our studies we investigated the interaction mode of different high affinity GlyT1 inhibitors with the transporter. We show that the compounds can be differentiated according to their competitive or non-competitive interaction with the glycine binding site. Using SSR504734 and its N-methyl derivative, we describe for the first time a competitive interaction of a high affinity GlyT1 inhibitor with the glycine binding site.

All tested compounds were potent inhibitors of glycine transport in native (mouse astrocytes) and recombinant systems expressing human GlyT1. Furthermore, all inhibitors, like glycine, blocked binding of both radioligands, [^{3}H]-(R)-NPTS and [^{3}H]-N-methyl-SSR504734 to recombinant GlyT1-expressing mammalian cell membranes, as well as to *Xenopus* oocyte membranes. For studying whether the compounds exert a competitive or non-competitive mode of inhibition, we employed two types of experiments. We first investigated the surmountability of inhibition of the electrogenic GlyT1 transport by high glycine levels. In these experiments, inhibition of SSR504734 and its N-methyl derivative were surmountable by high glycine, indicating that the inhibitor binding site is orthosteric to the site bound by glycine. The SSR504734-induced shift of the glycine concentration response curve by linear and non-linear Schild analysis confirmed that this compound was competitive for glycine. We also identified the sarcosine-based compounds as non-competitive inhibitors, as they exert inhibition independent of glycine concentration and are therefore non-surmountable. Because the inhibitor was applied under conditions of active transport, glycine could directly compete with the sarcosine-based inhibitor. Under these conditions, the apparently irreversible properties of these compounds should not play a role.

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These findings were corroborated with a second set of experiments where we studied the effects of the transport inhibitors on the binding parameters of two GlyT1 radioligands, [^{3}H]-(R)-NPTS and [^{3}H]-N-methyl-SSR504734. Glycine and SSR504734 shifted the K_d of [^{3}H]-N-methyl-SSR504734 to higher values, while not decreasing the B_{max}. This confirms the competitive interaction observed in the functional experiments and indicates that [^{3}H]-N-methyl-SSR504734 occupies a binding site orthosteric to the site bound by glycine. Inversely, as indicated by a decrease in B_{max}, the effects of these ligands revealed a non-competitive interaction on [^{3}H]-(R)-NPTS binding, indicating that this ligand binds to an allosteric site at the transporter. Contrary to the glycine-competing ligands, the sarcosine-based compounds increased the K_d for [^{3}H]-(R)-NPTS, consistent with a competitive inhibition of [^{3}H]-(R)-NPTS binding by these compounds. Correspondingly, sarcosine-based inhibitors decreased the B_{max} of the [^{3}H]-N-methyl-SSR504734, again indicative for a non-competitive interaction of this inhibitor class with the glycine binding site.

The finding that sarcosine-based compounds bind to a site distinct from the substrate (glycine) site corresponds to earlier reports (Aubrey and Vandenberg, 2001; Mallorga et al., 2003), describing a non-competitive interaction between the sarcosine-based GlyT1 inhibitor NFPS with glycine. In a more recent study, mutations of amino acids of the putative substrate (glycine) binding site in the transmembrane domain 6 (TM6) of the transporter affected substrate affinity and selectivity (glycine versus sarcosine affinity). However, these mutations only marginally decreased the affinity of NFPS (Vandenberg, 2006). In another study, employing GlyT1-GlyT2 chimeric transporters, the transmembrane domains TM1 and TM3 were found to be involved in NFPS binding (Núñez et al., 2005). Together, these data are consistent with a binding site for sarcosine-based structures distinct

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from the substrate binding site. Conversely, it has been reported earlier that sarcosine increased the K_d of [^{3}H]-(R)-NFPS binding and decreased the B_{max}. These data were interpreted as potentially overlapping binding sites of sarcosine and NFPS (Mallorga et al., 2003). Similar to sarcosine, our data with (R)-NPTS provide some hint for a potential dual binding of the sarcosine-based inhibitors, both to the allosteric binding site, and the orthosteric site. The reason for this interpretation is the increase of the K_d for the [^{3}H]-N-methyl-SSR504734 binding by (R)-NPTS. However, this effect could also be due to a non-competitive interaction affecting the ligand affinity, and therefore the binding of sarcosine-based compounds to the substrate binding site remains hypothetical. Furthermore, if there were any direct glycine-competitive interaction of the sarcosine-based compounds, its contribution to the overall compound affinity should be small, as mutations within sequences involved in glycine binding had only a small effect on NFPS binding (Vandenberg, 2006).

The major new finding in our studies is that SSR504734, as well as its N-methyl-derivative, are glycine-competitive inhibitors. This demonstrates that binding to the orthosteric and the allosteric binding sites is separable. Since the binding of all compounds to either site affects the B_{max} of ligands at the other site, the two sites appear to be closely coupled. This may be either a conformational coupling or an interaction based on spatial proximity. Compounds may bind to one site and prevent the binding of ligands to the other site by spatial hindrance.

The different modes of interaction described here may significantly impact the pharmacological profile of GlyT1 inhibitors. One important differentiating characteristic is the dependency of the degree of inhibition on the glycine concentration. While inhibition of GlyT1 by non-competitive inhibitors is not sensitive for the glycine concentration, obviously, the degree of inhibition of glycine-

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competitive inhibitors greatly depends on the concentration of glycine. The glycine concentration is extremely different between different compartments within the brain, as well as between different physiological situations. Competitive inhibitors may therefore exhibit a stronger blockade in the intra-synaptic compartment, where the glycine concentration is low, and a weaker inhibition in extra-synaptic compartments, where a higher concentration of glycine partly displaces the inhibitor. An argument in favor of a selective, synaptic blockade with glycine-competitive inhibitors is the neuron-specific GlyT1 knockout mouse (Yee et al., 2006; Singer et al., 2007; Sanderson and Bannermann, 2007). Because neuronal GlyT1 is predominantly localized in pre- and postsynaptic aspects of glutamatergic terminals (Cubelos et al., 2005), neuron-specific GlyT1 knockout affects mainly synaptic glycine transport. Such animals exhibit a more favorable pro-cognitive phenotype than mice with a global heterozygous GlyT1 knockout, which affects the neuronal and the astrocytic transporter. Therefore, compared to non-competitive inhibitors, gylcine-competitive inhibitors, having increased potential in the synaptic compartment, may be advantageous to improve cognitive performance in schizophrenia.

Competitive and non-competitive GlyT1 inhibitors may also exert different effects in situations with strongly increased glycine concentrations, such as ischemia or epileptic activities. Indeed, SSR504734 has been shown to be neuroprotective in a model of focal cerebral ischemia, while NPTS exacerbated ischemic damage in the same experiment (Szabo, 2005). So far the role of glycine in situations like ischemia is poorly understood, especially as it may have very complex effects involving activation of intrasynaptic and extrasynaptic NMDA receptors, as well as glycine A receptors. There is increasing evidence that intrasynaptic in contrast to extrasynaptic NMDA receptors provide a pro-survival signal for neurons (Soriano et al., 2006;

Zhang et al., 2007) and it is tempting to speculate that a differential effect on synaptic glycine may be responsible for the different effects of SSR504734 and NPTS in ischemia.

All considerations on the potential impact of the mode of interaction are, however, hypothetical, and need to be substantiated by experiments comparing this specific compound property. Sarcosine-based compounds are not suitable as representatives of non-competitive inhibitors, because apart from binding to an allosteric site, they are functionally apparently irreversible (Aubrey and Vandenberg, 2001; Atkinson et al., 2001). To investigate the impact of the mode of inhibition on the action of the drug *in vivo*, it is therefore necessary to identify non-competitive reversible GlyT1 inhibitors and to compare them with glycine-competitive and reversible GlyT1 inhibitors, like N-methyl-SSR504734 or SSR504734.

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Footnotes

# Authors contributed equally to the work.

a) Parts of the work were presented at: Mezler M, Hornberger W, van Gaalen MM, Wicke KM, Mueller R, Schmidt M, Braje W and Behl B (2005) Reversibility of glycine transporter GlyT1 inhibition comparing two chemical classes. *35th annual meeting of the Society of Neuroscience, Washington, DC*, Program No. 1022.9.

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Legends for figures

Figure 1: Two classes of GlyT1 inhibitors.

Chemical structure of sarcosine-based and non-sarcosine based GlyT1 inhibitors used in these studies. While (R)-NPTS, NFPS and Org24598 contain a sarcosine-moiety (marked light grey). This moiety is absent in SSR504734 and its N-methyl-derivative N-methyl-SSR504734.

Figure 2: SSR504734 is a reversible and surmountable inhibitor of GlyT1 transport.

(A) Inhibition of 3 µM SSR504734 was evaluated in the presence of 20 µM glycine (bold line) by addition of the inhibitor in the open state of the transporter (thin line). The compound blocked the glycine transport current with $t_{1/2on}$ = 11 ± 1 s (n=3) and inhibition could be reversed with 20 µM glycine at $t_{1/2off}$ = 40.2 ± 8.3 s (n=3). (B) Quantitative evaluation of glycine transport inhibition by SSR504734, calculated from C and D. While at 10 µM glycine the inhibitor blocked the glycine transport current by 82 ± 2 % (n=5), the glycine current of 3 mM glycine was only blocked by 36 ± 3 % (n=6). (C) GlyT1-expressing oocytes were stimulated with 20 µM glycine (1), 10 µM glycine (2) and 10 µM glycine + 3 µM SSR504734 (3; dashed line). (D) GlyT1-expressing oocytes were stimulated with 20 µM glycine (1); 3 mM glycine (2); 3 mM glycine + 3 µM SSR504734 (3; dashed line). Values are means ± SD, significance was tested by unpaired t-test, holding potential was –60 mV.

Figure 3: SSR504734 inhibition of GlyT1 is competitive for glycine transport in *Xenopus* oocytes.

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(A) Upper panel: concentration-response curve of glycine transport in a *Xenopus* oocyte expressing GlyT1c. Lower panel: the EC_{50} of glycine on GlyT1 in oocytes was 19.4 ± 5.7 µM (n=7). (B) Upper panel: concentration-response curve of glycine transport in an oocyte expressing GlyT1c. Besides a first and last 20 µM glycine stimulus (bold lines), at each concentration of the concentration-response determination, glycine was supplemented with 10 µM SSR504734 (thin lines). The EC_{50} value of glycine in the presence of 10 µM SSR504734 was right-shifted to 592 ± 40 µM (n=4). (C) Upper panel: Schild analysis of curve shifts with increasing concentrations of 1 µM, 3 µM and 10 µM SSR504734. Global fitting resulted in a calculated Schild slope of 1. Lower panel: accordingly, transformation into a linear Schild Plot resulted in a slope of 0.9 (bottom figure). The K_b determined from global non-linear fitting was 214 nM. N=4-7; holding potential was –60 mV.

Figure 4: N-methyl-SSR504734 inhibition is reversible and surmountable with high glycine concentrations.

(A) N-methyl-SSR504734 inhibition was evaluated in the presence of 20 µM glycine (bold bars) by addition of the inhibitor at 3 µM in the open state of the transporter (thin bar). The compound monophasically blocked the glycine transport current with $t_{1/2on}$ = 12.3 ± 0.3 sec (n=4) and inhibition could be reversed with 20 µM glycine at $t_{1/2off}$ = 868.4 ± 36 sec (n=4). (B) Quantitative evaluation of glycine transport inhibition by N-methyl-SSR504734, calculated from C and D. While at 10 µM glycine the inhibitor blocked the glycine transport current by 91 ± 5 % (n=5), the glycine current of 3 mM glycine was only blocked by 26 ± 4 % (n=4). (C) GlyT1-expressing oocytes were stimulated with 20 µM glycine (1), 10 µM glycine (2) and 10 µM glycine + 1 µM N-methyl-SSR504734 (3; dashed line). (D) GlyT1-expressing oocytes were

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stimulated with 20 µM glycine (1); 3 mM glycine (2); 3 mM glycine + 1 µM n-methyl-SSR504734 (3; dashed line). Due to the long transport times, for calculation of inhibition of (D), the reduction of the current with inhibitor was corrected for the reduction of the current with 3 mM glycine alone at the same time of wash-in. Values are means ± SD, significance: unpaired t-test, holding potential was –60 mV.

Figure 5: Sarcosine-containing compounds are apparently irreversible inhibitors of GlyT1.

Inhibition of NFPS, NPTS and Org24598 was evaluated in the presence of 20 µM glycine (bold bars) by addition of the inhibitor at 1 µM in the open state of the transporter (thin bar). A) 1 µM NFPS blocked the glycine transport current with $t_{1/2\ on}$ of 23 ± 4 s (n=4). B) 1 µM NPTS blocked the glycine transport current with $t_{1/2\ on}$ of 59 ± 4 s (n=4). C) 1 µM Org24598 blocked the glycine transport current with $t_{1/2\ on}$ 70 ± 2 s (n=4). For all three compounds the $t_{1/2\ off}$ could not be determined. Also a 2min washout with normal frog ringer solution did not reconstitute the transport activity, probed by application of 20 µM glycine.

Figure 6: Sarcosine-containing compounds are non-surmountable inhibitors of GlyT1 in *Xenopus* oocytes.

Oocytes expressing GlyT1c were stimulated with (A) 20 µM glycine (1); 10 µM glycine (2); 10 µM glycine + 500 nM NFPS (3, dashed line). (B) 20 µM glycine (1); 3 mM glycine (2); 3 mM glycine + 500 nM NFPS (3, dashed line). (C) Under both conditions - at 10 µM glycine and 3 mM glycine - NFPS strongly blocked the glycine-induced current by 91 ± 4 % (at 10 µM glycine) and 90 ± 4 % (at 3 mM glycine) respectively. (D) Under identical conditions inhibition of 2 µM NPTS at 10 µM and 3

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mM glycine was equal, at 96 ± 3 %. (E) 1.5 µM ORG24598 inhibited the glycine transport current at 10 µM by 93 ± 8 % and at 3 mM glycine by 96±1 %. Values in C, D and E are means ± SD, n=5 in all cases. The inhibition at 10 µM and 3 mM glycine current in Figures C, D and E were not statistically different (Student t-test). Holding potential was –60 mV.

Figure 7: Allosteric or competitive characteristics of GlyT1 inhibitors.

In saturation binding experiments of [^{3}H]-N-methyl-SSR504734 to recombinant human GlyT1c expressing CHO membranes, saturation binding isotherms were examined. Changes in saturation binding isotherms of [^{3}H]-N-methyl-SSR504734 with increasing compound concentrations demonstrate competitive displacement by SSR504734 (A) and glycine (B), but non-competitive displacement by (R)-NPTS (C). Changes in saturation binding isotherms of [^{3}H]-(R)-NPTS with increasing compound concentrations demonstrate non-competitive displacement by SSR504734 (D), glycine (E), and N-methyl-SSR504734 (F).

Graphs depict values (mean & SEM) and nonlinear regression lines (1-site model) from representative saturation binding experiments used for the calculations of the values given in table 2.

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Tables

Table 1: All compounds tested are potent inhibitors of GlyT1 and displace [^{3}H]-N-methyl-SSR504734 binding. Inhibition of [^{3}H]-glycine uptake by native mouse astrocytes (left column) and recombinant mammalian cells with stable expression of human GlyT1c (hGlyT1c_5_CHO cells; middle column). Cells were incubated with 200 nM of [^{3}H]-glycine and the uptake was measured after 90 or 120 min within the linear course of incorporation. Data represent geometric means and their 95% confidence limits (n = 2 to 6). All inhibitors tested, as well as the substrates glycine and sarcosine, compete for the binding of the new radioligand [^{3}H]-N-methyl-SSR504734 (right column).

	Inhibition of ^{3}H-glycine uptake IC_{50} and c.l. (nM)		[^{3}H]-N-methyl-SSR504734 displacement Ki_{app} and c.l. (nM)
	Mouse astrocytes	hGlyT1c_5_CHO	hGlyT1c_5_CHO membranes
NFPS / ALX5407	**0.18** (0.11 - 0.30)	**2.6** (1.9 – 3.7)	**3.5** (2.7 - 4.5)
(R)-NPTS	**0.29** (0.18 - 0.49)	**11.7** (7.6 - 18)	**13** (8.8 - 18)
Org24598	**3.4** (1.7 - 6.7)	**16** (10 – 24)	**259** (140 - 470)
SSR504734	**15** (9.6 – 22)	**314** (173 – 569)	**44** (44 - 55)
N-methyl-SSR504734	**1.6** (0.14 - 17)	**2.5** (1.8 – 3.4)	**7.5** (3.8 - 15)
Glycine	n.a.	n.a.	**760 µM** (550 – 1,000)
Sarcosine	**30,000** (5,400 – 170,000)	**5,600** (5,400 – 5,800)	**390 µM** (340 - 450)

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Table 2: Allosteric or competitive characteristics of GlyT1 inhibitors in saturation binding experiments of [^{3}H]-N-methyl-SSR504734 and [^{3}H]-(R)-NPTS to recombinant human GlyT1c expressing CHO membranes.

Upper panel: SSR504734 and glycine demonstrate competitive displacement of [^{3}H]-N-methyl-SSR504734 and increase the apparent K_d without relevant effect on the B_{max}. In contrast (R)-NPTS behaves as a non-competitive inhibitor.

Lower panel: Non-competitive displacement of [^{3}H]-(R)-NPTS binding was demonstrated for SSR504734 and N-methyl-SSR504734 by decrease of B_{max}, which was also observed as a trend for glycine.

Values represent geomeans and 95% confidence limits (N=2-4). ANOVA was used to assess significance of changes in K_d and B_{max} obtained from nonlinear regression analysis of 1-site saturation binding isotherms of the respective radioligand in the absence or presence of increasing concentrations of test compound; values without confidence limits were not included in the ANOVA analysis.

Radioligand	**[^{3}H]-N-methyl-SSR504734**					
Compound	**SSR504734**		**Glycine**		**(R)-NPTS**	
Concentration	Kd (nM) CL	Bmax (pmol/mg) CL	Kd (nM) CL	Bmax (pmol/mg) CL	Kd (nM) CL	Bmax (pmol/mg) CL
Control	3.5 (2.2 - 5.6)	15.4 (6.7 - 35)	2.0 (0.1 - 45)	21.5 (8.8 - 52)	5.3 (1.9 - 14.4)	19.8 (11.8 - 33)
1 nM					6.5	20.3
10 nM	4.7	9			6.6 (1.0 - 46)	16.8 (9.0 - 31)
30 nM	4.9 (2.8 - 8.5)	12.1 (5.9 - 25)			11.8	15
100 nM	9.2 (6.3 - 13)	12.1 (5.4 - 27)			21 (0.6 -753)	11.7 (1.2 -113)
300 nM	28 (18 - 43)	13.3 (5.3 - 33)				
1000 nM	107 (39 - 293)	20.7 (4.4 - 98)			32 (14 - 71)	5.9 (4.9 - 7.1)
0.3 mM			3.4 (0.1 - 96)	23 (13.2 - 40)		
1 mM			5.3 (0.7 - 40)	21 (8.8 - 52)		
3 mM			9.5 (4.9 - 19)	21 (9.0 - 52)		
10 mM			20 (1.2 - 344)	24		
ANOVA	P<0.0001	ns	P=0.0076	ns	P=0.0237	P=0.018
Binding mode	**competitive**		**competitive**		**non-competitive (Kd-type)**	

Radioligand	**[^{3}H]-(R)-NPTS**					
Compound	**SSR504734**		**Glycine**		**N-methyl-SSR504734**	
Concentration	Kd (nM) CL	Bmax (pmol/mg) CL	Kd (nM) CL	Bmax (pmol/mg) CL	Kd (nM) CL	Bmax (pmol/mg) CL
Control	4.3 (1.8 - 10.2)	15.6 (3.6 - 68)	1.8 (0.4 - 71)	8.4 (4.7 - 15)	3.0 (0.3 - 30)	9.4 (6.9 - 13)
1 nM						
10 nM					3.9 (0.7 - 22)	7.3 (5.0 - 11)
30 nM	2.1 (0.8 - 5.4)	9.4 (1.8 - 50)				
100 nM	3.1 (1.7 - 5.6)	8.9 (2.1 - 38)			3.5 (0.5 - 21)	5.2 (3.5 - 7.8)
300 nM	2.7 (0.9 - 8.2)	6.1 (1.0 - 37)			3.4 (0.5 - 22)	4.5 (2.9 - 6.9)
1000 nM	4.0 (0.3 - 52)	5.1 (0.1 - 180)			4.7 (0.4 - 52)	2.9 (0.7 - 12)
0.3 mM			2.3 (0.3 - 17)	8.7 (3.6 - 21)		
1 mM			3.4 (1.6 - 73)	7.6 (4.0 - 14)		
3 mM			3.6 (1.0 - 130)	7.4 (0.6 - 88)		
10 mM			8.4 (0.4 - 206)	6.1 (0.4 - 84)		
ANOVA	ns	P=0.007	ns	ns	ns	P=0.001
Binding mode	**non-competitive**		**(non-competitive)**		**non-competitive**	

Figure 1

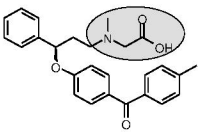
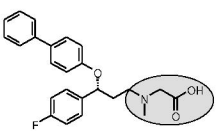
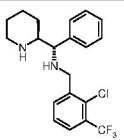
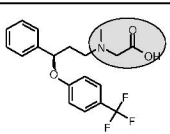
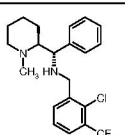
	(R)-NPTS		Sarcosine
	NFPS ALX5407		SSR504734
	Org24598		N-Methyl-SSR504734

Figure 2

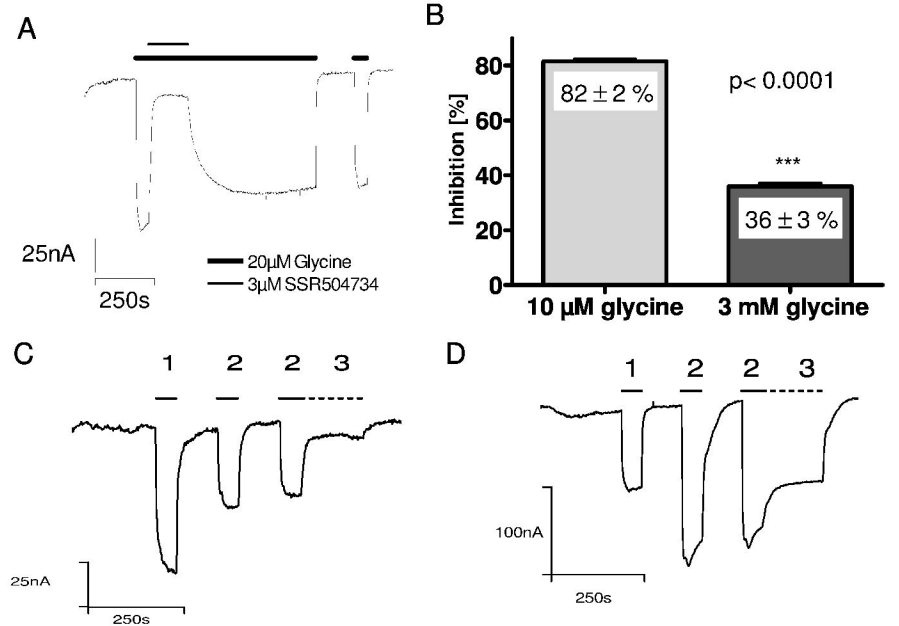

A
25nA
250s
20μM Glycine
3μM SSR504734
B
Inhibition [%]
80
60
40
20
0
82 ± 2 %
36 ± 3 %
p< 0.0001
***
10 μM glycine
3 mM glycine
C
1
2
2
3
25nA
250s
D
1
2
2
3
100nA
250s

Figure 3

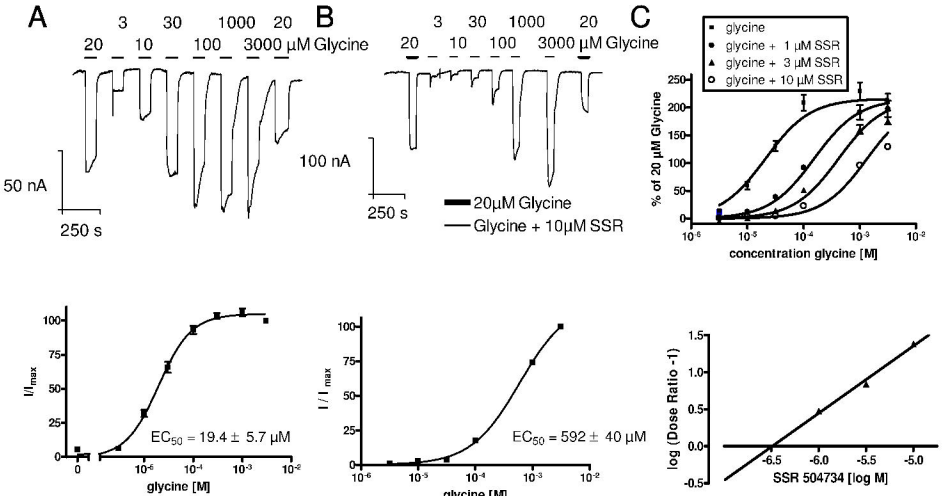

A
3 30 1000 20
20 10 100 3000 µM Glycine
50 nA
250 s
B
3 30 1000 20
20 10 100 3000 µM Glycine
100 nA
250 s
20µM Glycine
Glycine + 10µM SSR
C
glycine
glycine + 1 µM SSR
glycine + 3 µM SSR
glycine + 10 µM SSR
% of 20 µM Glycine
concentration glycine [M]
I/I max
EC50 = 19.4 ± 5.7 µM
glycine [M]
I / I max
EC50 = 592 ± 40 µM
glycine [M]
log (Dose Ratio -1)
SSR 504734 [log M]

Figure 4

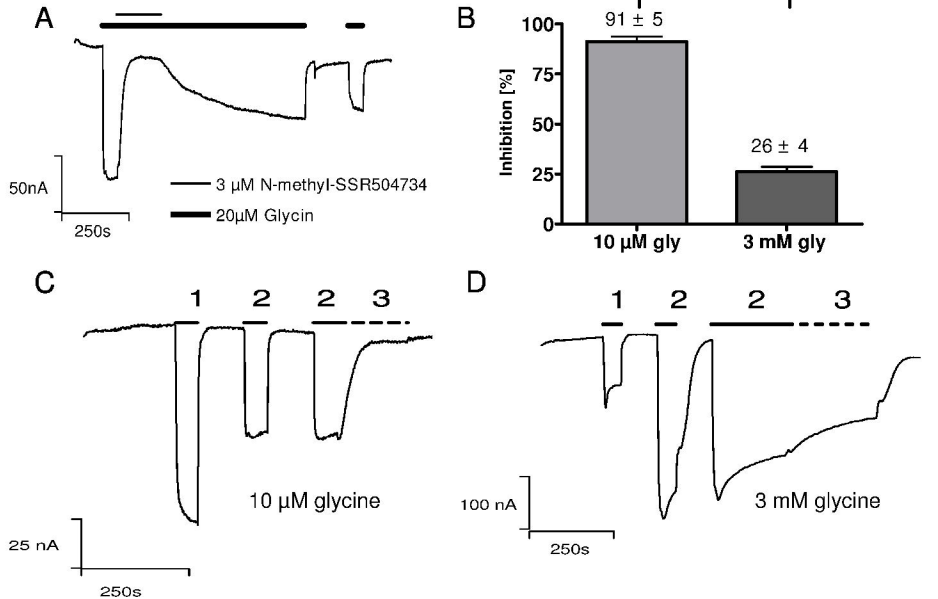

A
50nA
250s
3 µM N-methyl-SSR504734
20µM Glycin
B
p<0.0001
91 ± 5
26 ± 4
Inhibition [%]
100
75
50
25
0
10 µM gly
3 mM gly
C
1
2
2
3
10 µM glycine
25 nA
250s
D
1
2
2
3
3 mM glycine
100 nA
250s

Figure 5

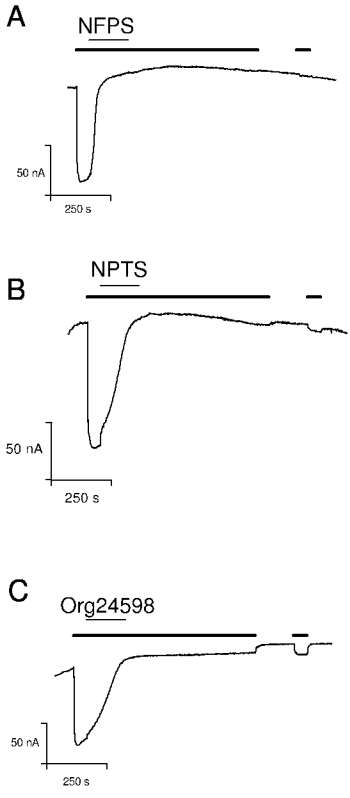


Figure 6

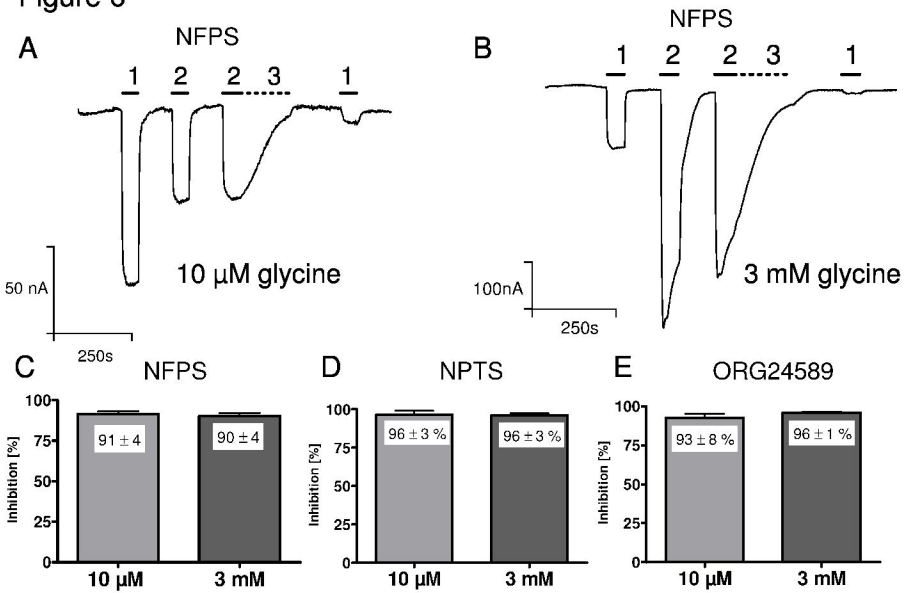
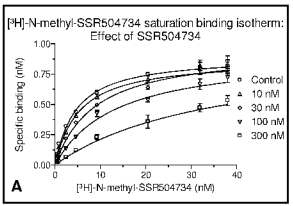
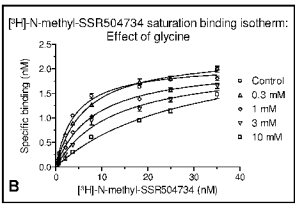
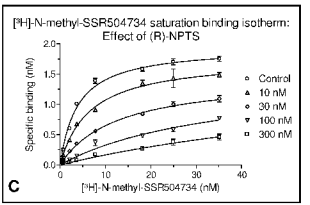
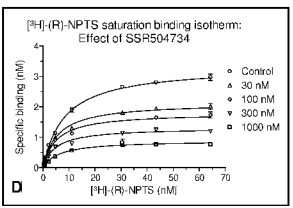

A
NFPS
1
2
2
3
1
50 nA
250s
10 µM glycine
B
NFPS
1
2
2
3
1
100nA
250s
3 mM glycine
C
NFPS
Inhibition [%]
91 ± 4
90 ± 4
10 µM
3 mM
D
NPTS
Inhibition [%]
96 ± 3 %
96 ± 3 %
10 µM
3 mM
E
ORG24589
Inhibition [%]
93 ± 8 %
96 ± 1 %
10 µM
3 mM

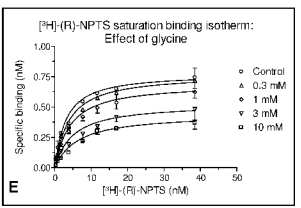
Figure 7

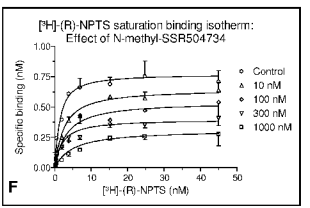

[³H]-N-methyl-SSR504734 saturation binding isotherm:
Effect of SSR504734
Specific binding (nM)
[³H]-N-methyl-SSR504734 (nM)
Control
10 nM
30 nM
100 nM
300 nM
A


[³H]-N-methyl-SSR504734 saturation binding isotherm:
Effect of glycine
Specific binding (nM)
[³H]-N-methyl-SSR504734 (nM)
Control
0.3 mM
1 mM
3 mM
10 mM
B


[³H]-N-methyl-SSR504734 saturation binding isotherm:
Effect of (R)-NPTS
Specific binding (nM)
[³H]-N-methyl-SSR504734 (nM)
Control
10 nM
30 nM
100 nM
300 nM
C


[³H]-(R)-NPTS saturation binding isotherm:
Effect of SSR504734
Specific binding (nM)
[³H]-(R)-NPTS (nM)
Control
30 nM
100 nM
300 nM
1000 nM
D


[³H]-(R)-NPTS saturation binding isotherm:
Effect of glycine
Specific binding (nM)
[³H]-(R)-NPTS (nM)
Control
0.3 mM
1 mM
3 mM
10 mM
E


[³H]-(R)-NPTS saturation binding isotherm:
Effect of N-methyl-SSR504734
Specific binding (nM)
[³H]-(R)-NPTS (nM)
Control
10 nM
100 nM
300 nM
1000 nM
F