Glycine Site Modulators and Glycine Transporter-1 Inhibitors as Novel Therapeutic Targets for the Treatment of Schizophrenia

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Abstract: Current antipsychotic medications are efficacious for the positive symptoms of schizophrenia. However, there remains a significant unmet need for alternate strategies that could result in improved tolerability and/or efficacy for negative and cognitive symptoms. A growing body of research suggests that NMDA mediated neuronal activity is involved in the etiology of schizophrenia. Glycine binds to a modulatory glycine$_B$ strychnine-insensitive binding site on the NR1 subunit of the NMDA receptor complex and acts necessary co-agonist for activation of the NMDA receptor. Thus, several approaches have emerged aimed towards modulating this glycine binding site. To date, the glycine$_B$ site agonists glycine and D-serine, the partial agonist, D-cycloserine and the glycine reuptake inhibitor, sarcosine, have been shown to provide relief to schizophrenic patients. These clinical findings, combined with a growing body of preclinical literature, support the notion that enhancing synaptic glycine$_B$ activity leads to an increase in the effectiveness of normal glutamatergic signaling at the NMDA receptor complex and provides efficacy for schizophrenic patients. Accordingly, the present review examines the role of glycine$_B$ site modulation as a therapeutic approach for the treatment of schizophrenia.

INTRODUCTION

Schizophrenia is a heterogeneous disorder characterized by positive (paranoia, hallucinations, delusions) and negative (blunted affect, withdrawal, anhedonia) symptoms in addition to cognitive dysfunction. Existing therapies for the treatment of schizophrenia share as a common mechanism their ability to antagonize dopamine D_2 receptors. This strategy stems from the dopamine hypothesis of schizophrenia, which attributes excessive dopaminergic transmission in the forebrain as a key causative factor in the disease. While both typical and atypical antipsychotic drugs are generally effective in treating the positive symptoms of schizophrenia, residual negative and cognitive symptoms typically remain. Further, current antipsychotics are associated with a variety of side effects including tardive dyskinesia, sedation, weight gain and sexual dysfunction. Thus, the need for improved therapeutics remains.

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system with glutamate immunoreactive neurons representing nearly 50% of cortical neuronal populations [14]. Glutamate exerts its actions through a variety of ionotropic and metabotropic receptors (iGluR and mGluR, respectively) that are distributed both pre and post-synaptically throughout neuronal populations in the brain. The rationale for use of ligands interacting with mGluRs as putative therapeutics for schizophrenia is reviewed elsewhere in this edition (see e.g., Chavez-Noriega and Conn). One class of iGluR is the *N*-methyl-D-aspartate receptor (NMDAR) complex. The NMDAR is distinguished from other iGluRs by several unique properties. Among these is dependence on depolarization in order to relieve a Mg^{2+} block of the channel, a complex subunit makeup that provides for functional heterogeneity and a requirement for co-activation of a glutamate binding site and a strychnine insensitive glycine binding site (i.e., the glycine$_B$ site).

The NMDAR has been implicated in schizophrenic conditions following the observation that administration of use-dependent NMDAR antagonists induced a variety of symptoms that resemble those observed in the clinic [31, 46]. Although a variety of psychoactive drugs are known to induce psychosis, including psychomotor stimulants (cocaine, amphetamine), hallucinogens (LSD), and NMDA receptor antagonists (PCP, ketamine), only NMDA-receptor antagonists appear to reproduce the full spectrum of symptoms observed in

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schizophrenia [31, 46]. In addition to producing these symptoms in normal humans, NMDAR antagonists can also exacerbate existing symptoms in schizophrenics and trigger the re-emergence of sym-ptoms in stable patients [51]. These observations suggest that hypo-function of NMDARs may be a critical component of schizophrenia (i.e., the NMDA receptor hypofunction hypothesis). This hypothesis is further supported by the finding of increased NMDAR density in multiple brain regions of schizophrenic patients [29, 30]. The corollary of the NMDAR hypofunction hypothesis suggests that any drug capable of potentiating NMDAR activity in a physiologically relevant manner may ameliorate schizophrenic symptoms.

Glycine is a well-characterized simple amino acid inhibitory and excitatory neurotransmitter [1, 36]. As an inhibitory neurotransmitter, glycine acts at the ligand-gated, strychnine sensitive $glycine_A$ binding site in cerebellum, brainstem and spinal cord [1, 6]. As an excitatory neurotransmitter, glycine interacts with the $glycine_B$ site in forebrain areas like cortex, hippo-campus and thalamus [5, 13, 36], and activation of this $glycine_B$ binding site on the NR1 subunit of the NMDAR complex is a necessary component for subsequent glutamate binding to the NR2 subunit of the receptor complex (Fig. **1**). Interestingly, the affinity of glycine for the $glycine_B$ binding site can vary between 0.1 to 3.0 μM depending on the NR2 isoform makeup of the NMDAR complex [see 13]. When a non-essential NR3 subunit co-assembles with NR1 the NMDAR complex behaves as an excitatory glycine receptor that is insensitive to glutamate. Further, NR3/NR1 containing NMDAR complexes are inhibited by D-serine, which normally acts as an agonist at the $glycine_B$ binding site [9]. These findings suggest that glycine serves a modulatory role on NMDAR function whose sensitivity is dependent on regional NMDAR subunit expression. The modulatory nature of this interaction has led several groups to hypothesize that enhancing the activity at the $glycine_B$ site will allow for an increase in normal glutamateric signaling at the NMDAR while maintaining the fidelity of this signaling in a regionally specific manner thereby decreasing the likelihood of toxicity that has been associated with glutamate receptor agonism.

Several strategies have been employed as therapeutic approaches to potentiate NMDAR function *via* activation of the $glycine_B$ binding site. These include (1) direct interaction with the ligand binding site using agonists such as glycine and D-serine, (2) blockade of the glycine reuptake site, GlyT1, in order to increase synaptic glycine levels, (3) prevention of the degradation of D-serine, and (4) enhancing conversion of L-serine to D-serine. These approaches are discussed below.

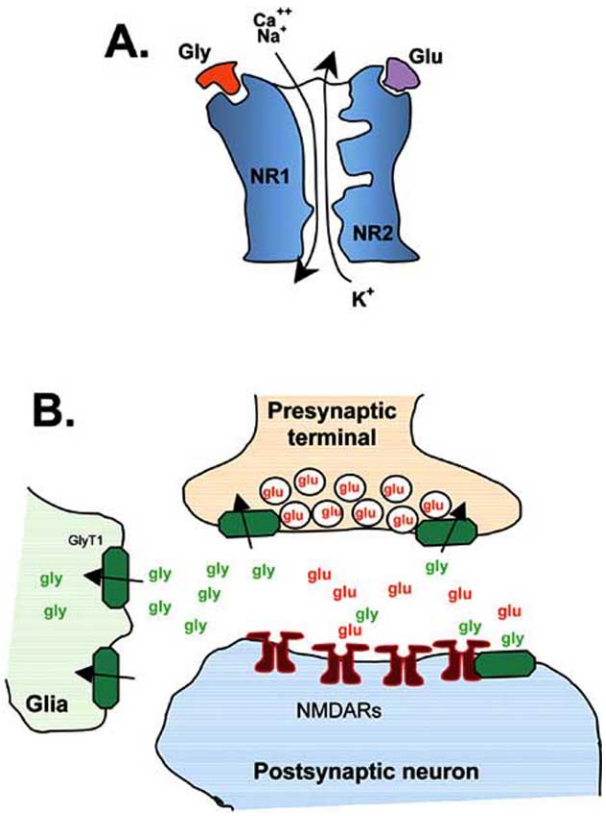

Fig. (1). (A) A schematic representation of the NMDA receptor complex. Note the requirement for binding of glycine (Gly) to the NR1 subunit of this receptor as a necessary component of glutamatergic signaling (Glu). Following relief of Mg2+ block by depolarization and subsequent Gly binding, Glu activates this receptor complex through binding to the NR2 subunit allowing for ionic conductance. (B) A schematic representation of an NMDA receptor containing excitatory synapse. In this drawing, Glia containing the glycine transporter, GlyT1, are localized in close proximity to the synaptic region. This proximity allows for close regulation of synaptic glycine. Direct application of glycine site agonists and inhibition of GlyT1 activity are two approaches that have been considered to enhance NMDA receptor function while maintaining the fidelity of normal Glu signaling.

GLYCINE SITE ACTIVATORS

Antipsychotic efficacy following activation of the $glycine_B$ binding site has been demonstrated in multiple clinical trials. Direct activators of the $glycine_B$ binding

site include glycine and D-serine (Fig. **2**) [22]. D-cycloserine represents a partial agonist at the glycine$_B$ binding site (Fig. **2**) [53]. Several studies evaluating the therapeutic benefit of glycine in stably treated schizophrenic populations have demonstrated significant therapeutic effects on positive, negative and cognitive symptoms [24, 25, 28, 32, 34]. Interestingly, efficacy on negative symptoms, cognition and depression in the PANSS scores were correlated with a ~3.5 fold increase in serum glycine levels for treated patients relative to control patients [25]. It is notable that these studies utilized antipsychotic-treated patients. Thus, the possibility that positive symptoms in some trials were relatively well controlled prior to glycine treatment must be considered making the finding of a significant improvement in positive symptoms relatively impressive [28, 34]. It is further interesting that glycine treatment is not efficacious in clozapine treated patients [16]. The possibility that this lack of effect in clozapine treated patients is due to an interaction of clozapine with amino acid transporters resulting in enhanced synaptic glycine availability is considered below.

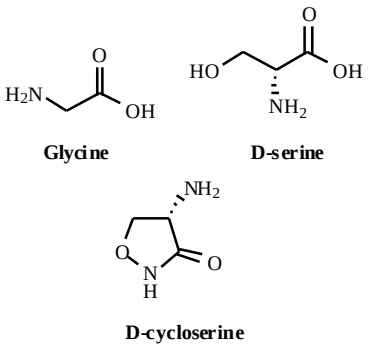

Fig. (2). Chemical structures of the glycine$_B$ site activators, glycine, D-serine and the partial agonist D-cycloserine.

In addition to the increase in serum glycine observed following glycine administration, serum serine levels are also increased [25]. Since D-serine is a more potent agonist than glycine at the glycine$_B$ site and has a greater ability to penetrate the blood brain barrier [22] this finding suggested that D-serine administration may also have a beneficial effect in schizophrenic patients. Tsai and colleagues [59] first tested this hypothesis in a double-blind, placebo-controlled trial in 29 Taiwanese schizophrenic patients. In that study, D-serine (30 mg/kg/day) was added to ongoing antipsychotic treatment for 6 weeks. Treatment resulted in a significant improvement of positive, negative and cognitive symptoms that was significantly correlated with increases in serum D-serine levels [59]. This initial finding was recently replicated in patients undergoing olanzapine and/or risperidone therapy [27], but not in clozapine treated patients [60].

D-cycloserine has also been evaluated in the clinical setting. Due to its partial agonist activity, it is not surprising that this compound results in an efficacious response with a U-shaped dose response function. Thus, at doses of approximately 50 mg/day an efficacious response on negative symptoms has been noted, however, at 250 mg/day this efficacy is lost [19, 26]. The efficacy of D-cycloserine is well correlated with low baseline serum glycine levels and enhanced serum glycine during treatment. It is of interest that patients taking clozapine demonstrate a worsening of symptoms following treatment with D-cycloserine with a U-shaped dose response function [18]. This finding, taken with the lack of efficacy of glycine and D-serine in clozapine treated patients, suggests that clozapine may influence synaptic glycine levels. Thus, D-cycloserine would be expected to act as a functional antagonist if this site were fully occupied through direct action of clozapine. In support of this possibility, two recent studies have found that Clozapine can directly influence both GlyT1 activity and System A mediated transport. Williams *et al*. [65] have recently reported that clozapine inhibits human GlyT1 activity in a non-competitive manner. In this study, it was estimated that therapeutic doses of clozapine may block ~30% of GlyT1 activity in brain. Further, Javitt and colleagues [35] demonstrated that clozapine can directly inhibit System A mediated transport of glycine and 2-methyl-aminoisobutyric acid suggesting that clozapine may interfere with System A mediated transport of amino acids and further suggesting that the role of System A transport must be considered when evaluating the inhibition of glycine reuptake as a therapeutic strategy.

GLYCINE TRANSPORTER BLOCKADE

Blockade of transporters that are specific for glycine represents an additional approach to increase activity at the glycine$_B$ site by increasing synaptic glycine levels. It is currently unclear whether the needed effect is an increase in the absolute amount of glycine available to bind to NMDARs or if a reduction of the kinetics of clearance of glycine from the synaptic region are more important, or a combination of the two. Nonetheless, selective blockade of neurotransmitter reuptake has proven clinically successful for the treatment of other diseases such as depression and anxiety. Thus, this

approach appears to provide a rational means towards use-dependent enhancement of NMDAR function.

GlyT1 and GlyT2 have been identified as two transporters that modulate glycine reuptake [7, 37, 42, 47, 54]. These transporters belong to the 12-transmembrane domain Na^+/Cl^- dependent family of neurotransmitter transporters which includes taurine, γ-aminobutyric acid (GABA), proline and monoamine transporters. Both forms of transporter are known to exist in multiple isoforms (GlyT1a-d and GlyT2a-c, respectively), which may ultimately underlie differential expression and developmental regulation [15, 37]. Although GlyT1 and GlyT2 show high inter-species homology [50] they are only ~50% homologous to each other, display differential regional distribution and underlie differential functional activity. GlyT1 is found relatively ubiquitously throughout the forebrain while GlyT2 is localized in the brain stem and cerebellum [54, 66, 67]. At the cellular level, GlyT2 is colocalized with glutamic acid decarboxylase (GAD) and expressed at glycinergic nerve endings in the spinal cord, brainstem and cerebellum [54, 66, 67]. In addition to an overlapping distribution with GlyT2, GlyT1 is highly expressed by glial cells in areas of the cortex, hippocampus, septum and thalamus, is found presynaptically in glutamatergic nerve terminals in forebrain regions, and is also found in postsynaptic densities physically associated with NMDARs [12, 54]. This latter finding has led researchers to the suggestion that GlyT1 may be optimally positioned to modulate glycine concentration in NMDAR expressing synapses, whereas GlyT2 may be primarilary responsible for regulation of extrasynaptic glycine at glycinergic synapses (i.e., $glycine_A$ containing synapses).

GlyT1 and GlyT2 have also been differentiated pharmacologically. Thus, sarcosine (N-methylglycine) acts as a selective GlyT1 substrate with functional antagonist activity in glycine uptake assays [37, 42, 54, 55]. Although sarcosine is selective for inhibition of GlyT1 over GlyT2, this compound may also interact with System A transporters [see 35]. Nonetheless, studies using sarcosine suggested that pharmacological agents could be further developed to specifically interact with GlyT1 in order to test the concept that potentiation of NMDAR activity *via* blockade of GlyT1 is tenable. Recent studies have described the sarcosine derivatives, NFPS ((*N*-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl])sarcosine) [2, 3, 5, 43], Org 24598 (*R*-(-)-*N*-methyl-*N*-[3-[(4-trifluoromethyl) phenoxy]-3-phenyl-propyl]glycine) [8] and Org 24461 (*R,S*-(+/-)-*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propylglycine) [21] as potent and selective GlyT1 inhibitors (Fig. **3**). Recent *in vitro* and *in vivo* studies using these compounds have supported the view that selective blockade of GlyT1 may promote NMDAR mediated functional activity (see below). Additionally, a great deal of pharmaceutical industry interest has led to the development of additional sarcosine-based and non-sarcosine based GlyT1 inhibitors [see 56]. Although the potency and efficacy of many of these novel GlyT1 inhibitors are not known in detail, the multiplicity of structures and number of pharmaceutical companies involved highlights the intense activity and interest in GlyT1 inhibitor research.

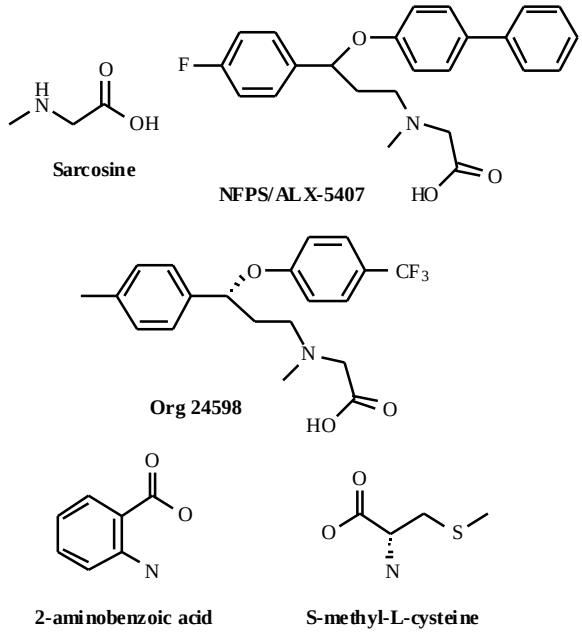

Fig. (3). Chemical structures of: (1) the glycine transporter type-1 (GlyT1) inhibitors, sarcosine, NFPS [2], and Org 24598 [8]; and (2) compounds reported to inhibit D-amino acid oxidase activity (viz., 2-amiobenzoic acid [48]) and reported to inhibit asc-1 transporter activity (viz., S-methyl-L-cysteine [57]).

In vitro experiments in rat hippocampal and brainstem slices demonstrated that blockade of GlyT1 with NFPS potentiates NMDA receptor-mediated excitatory post-synaptic currents [4, 5]. Subsequent experiments have shown that NMDAR activity is enhanced following selective blockade of GlyT1 *in vivo* [10, 38]. Chen *et al*. [10] demonstrated that NFPS both potentiated NMDA mediated responses and reversed a reduction of NMDA response in prefrontal cortical neurons produced by local application of a specific glycine site antagonist, (+)HA-966. Further,

systemic NFPS administration potentiated long-term potentiation (LTP) in the dentate gyrus of the hippocampus and increased c-fos expression in neurons of the nucleus accumbens, two physiological responses known to depend upon activation of NMDA receptors [38]. Collectively these observations suggest that NMDA receptor-mediated excitatory neurotransmission can be potentiated by selective GlyT1 antagonists and that synaptic levels of glycine are maintained at subsaturated concentrations by GlyT1 in NMDAR containing synapses *in vivo*.

Additional evidence comes from preclinical behavioral studies using assay conditions (a) sensitive to NMDAR antagonists and (b) sensitive to anti-psychotic drug treatment. Early studies using glycyldo-decylamide (GDA) demonstrated a selective reversal of PCP-induced locomotor activity in mice [33, 58]. More recent studies using the selective GlyT1 inhibitor, NFPS, demonstrated a potentiation of prepulse inhibition (PPI) of the acoustic startle response with a magnitude similar to clozapine in DBA/2J mice [38], a strain that demonstrates low basal levels of PPI [45]. NFPS and Org 24461 have also inhibited PCP and amphetamine induced locomotor activity in mice and PCP induced changes in rat EEG [21]. Finally, glycine and Org 24598 are effective in reversing amphetamine induced locomotor activity and PPI deficits in neonatal ventral hippocampally lesioned rats [40], an animal model resulting in a developmentally regulated psychotic-like phenotype [41].

Studies using GlyT1 heterozygotic knockout mice suggest that a 50% reduction of glycine reuptake is sufficient for an efficacious response in rodents and that this level of reuptake inhibition is generally well-tolerated. Heterozygotic GlyT1 knockout mice display a ~50% reduction in glycine re-uptake and are devoid of any gross deleterious behavioral phenotype [20, 62]. Nonetheless, the 50% reduction of glycine reuptake resulted in saturation of glycine at the NMDAR as demonstrated by an inability of exogenous glycine application to enhance NMDAR currents at hippo-campal synapses. These heterozygotes also showed improved performance during the probe trial of a water maze task and a reversal or augmentation of amphetamine and MK-801 induced deficits in PPI, respectively [62]. The differential effects of GlyT1 reduction on amphetamine versus MK-801 induced deficits suggests that excess synaptic glycine in the heterozygotic mice increases the ability of NMDAR open-channel blockers, such as MK-801, to access their site of action. These results from genetically engineered mice mirror the lack of significant adverse events in clinical trials employing glycine$_B$ site agonist or sarcosine potentially reflecting the modulatory role of the glycine$_B$ binding site on NMDAR function. Further investigation is needed to understand why an enhancement of PCP induced activity (PPI) was noted in the knockout mouse while reversal of PCP activities (locomotor activity and EEG effects) have been reported following administration of GDA and Org 24461.

Collectively, research focused on modulators of GlyT1 suggests that inhibitors of this transporter potentiate NMDAR mediated function and result in rodent behavior similar to those of known antipsychotic agents. These data further indicate that blockade of GlyT1 may represent a useful clinical approach towards the treatment of schizophrenia. In a recent 6-week double blind, placebo-controlled trial stably treated schizophrenic patients were treated with the GlyT1 inhibitor sarcosine (2 g/day). Supporting an efficacious role for GlyT1 inhibition in the treatment of schizophrenia the results from this trial demonstrated a significant and efficacious response on negative, positive and cognitive symptoms [61].

D-SERINE APROACHES: DAAO, SERINE RACEMASE AND D-SERINE TRANSPORTER (ASC-1)

In addition to approaches aimed at modulation of glycine, D-serine modulation has also been proposed as a possible target for pharmacological intervention. Chumakov and colleagues [11] recently demonstrated a genetic-link of the primate specific G72 gene and schizophrenia in Canadian and Russian populations. Supporting reports for association of this gene locus, or polymorphisms therein, and schizophrenia have been subsequently described in Chinese [64], German [52] and Ashkenazi Jewish populations [39]. G72 positively modulates D-amino acid oxidase (DAAO), which degrades D-serine. Thus, a gain in function in, or overexpression of, G72 could lead to increased degradation of D-serine and subsequent deficient activation of the glycine$_B$ site by endogenous D-serine. A relevant finding is the recent description of a trend towards an increase in G72 overexpression in postmortem dorsolateral prefrontal cortex from schizophrenic patients [39]. Consistent with the suggestion that inhibition of DAAO may enhance D-serine mediated activity at the NMDAR, mutant mice

lacking DAAO show enhanced NMDAR-mediated electrophysiological responses [63]. Increased patent activity and public disclosures suggest that multiple companies (e.g., Serono/ Genset, Sepracor, etc.) are beginning to evaluate the therapeutic opportunities of DAAO inhibitors and several such inhibitors have been disclosed in a recent patent publication (e.g., 2-aminobenzoic acid, Fig. **3**) [48]. No functional activity of these compounds has yet been discussed. In addition to inhibiting the breakdown of D-serine, promoting the conversion of L-serine to D-serine by enhancing serine racemase activity could be envisioned. At present, however, the public disclosure of such compounds is lacking.

A third approach towards enhancing D-serine is the blockade of D-serine reuptake in a manner similar to the approach outlined above for GlyT1. The alanine-serine-cysteine transporter-1 (asc-1) has been cloned and described as a Na^+-independent transporter with high affinity for D-serine [17, 49]. Asc-1 shows widespread neuronal distribution in the rodent brain with presynaptic, dendritic and somatic localization [23, 44]. The lack of glial distribution for asc-1 suggests that this transporter is well positioned for the removal of D-serine from the synaptic region. Thus, an unknown mechanism and/or transporter remains to be elucidated to account for the release of D-serine from glia. *S*-methyl-L-cysteine (Fig. **3**) has been described as an inhibitor of asc-1 and following local application into the ventral hippocampus, increases in the level of serine, alanine, threonine and glycine are observed [57]. These data are supportive of the notion that asc-1 inhibitors may elevate synaptic D-serine levels at NMDAR containing synapses and suggest that further study towards the development of asc-1 inhibitors are warranted.

CONCLUSION

Despite major advances in antipsychotic medications key unmet needs remain. Chief among these is a need for increased tolerability and efficacy both in terms of treatment of non-responders and efficacy against negative and cognitive symptoms. NMDA receptor hypofunction represents an alternate hypothesis, albeit not necessarily exclusive, to the prevailing dopamine hypothesis of schizophrenia.

In the present review, rationale and evidence for activation of the $glycine_B$ binding site, inhibition of GlyT1, inhibition of DAAO and inhibition of asc-1 as approaches to enhance NMDAR function are discussed. Evidence from both preclinical and clinical studies suggests an antipsychotic profile following $glycine_B$ stimulation that may ultimately prove useful for positive, negative and cognitive symptoms. While the most clinically advanced evidence currently exists for the use of $glycine_B$ agonists, such as glycine and D-serine, alternate approaches are also progressing due, in part, to the poor brain penetration and poor pharmacokinetic properties of these amino acids. The potential utility of GlyT1 inhibitors for schizophrenia has received increasing preclinical attention and a recent clinical study evaluating the activity of sarcosine in schizophrenic patients provides further evidence that inhibition of GlyT1 results in efficacy in a clinical setting. Rationale also exists for potentiation of D-serine activity by decreasing inhibition (DAAO inhibition), promoting synthesis (serine racemase), and/or decreasing uptake (asc-1 inhibition). Although relatively little data exists to fully evaluate these D-serine based approaches, recent patent activity suggests an increasing level of interest in these approaches. The clinical efficacy of D-serine exogenous administration further supports these as rationale approaches towards the evaluation of new therapeutics.

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