THE ROLE OF THE GLUTAMATERGIC SYSTEM IN PSYCHIATRIC
BEHAVIORAL ENDOPHENOTYPES IN MICE:
IMPLICATIONS FOR SCHIZOPHRENIA

By

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy,
Graduate Department of the Institute of Medical Science,
University of Toronto

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The role of the glutamatergic system in psychiatric behavioral endophenotypes in mice: implications for schizophrenia

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Institute of Medical Science, University of Toronto

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ABSTRACT

Reduced activity of the $N$-methyl-$d$-aspartate receptor (NMDAR) has been implicated in the pathophysiology of schizophrenia. The NMDAR contains a glycine site on the NR1 subunit that may be a promising therapeutic target for psychiatric illness. Recently, D-serine has been discovered to be a high-affinity endogenous activator of the NMDAR glycine site. Levels of D-serine in the brain are controlled by its synthesis enzyme serine racemase (Srr) and its catabolic enzyme D-amino acid oxidase (DAO). This work investigates the NMDAR glycine site, D-serine, and D-serine-regulatory enzymes Srr and DAO in the pathophysiology and treatment of symptomatology relevant to schizophrenia and other psychiatric disorders. Pharmacological and genetic mouse models were used to alter glycine site function and D-serine availability. Behavioral responses in these models were assessed. Administration of exogenous D-serine and the glycine transporter 1 (GlyT-1) inhibitor ALX-5407 improved performance of C57BL/6J mice in behavioral tests examining prepulse inhibition (PPI) or latent inhibition (LI). These compounds also reversed impairments induced by the NMDAR antagonist MK-801, and produced similar beneficial effects to the classical atypical antipsychotic clozapine. Mice carrying a point mutation that leads to diminished NMDAR glycine site function demonstrated abnormally persistent LI
and deficits in social approach and spatial recognition that were reversible by D-serine or clozapine administration. Similarly, mutant mice that lacked Srr function and had a severe reduction in D-serine displayed impairments in sociability, PPI, spatial recognition and memory. Behavioral deficits in mice without Srr were exacerbated by MK-801 and rescued by treatment with D-serine or clozapine. A genetically-induced loss of DAO function in mice resulted in the elevation of brain D-serine levels, and produced improvements in spatial reversal memory and extinction of a learned response in the Morris water maze, consistent with the effects of exogenous D-serine application in wild-type mice. Thus, deficiencies in NMDAR glycine site function and D-serine availability produce behavioral disturbances that are relevant to the negative and cognitive symptoms of schizophrenia. Activation of the NMDAR glycine site by D-serine, GlyT-1 inhibition, or diminished DAO activity may be beneficial for the treatment of schizophrenia and other psychopathologies involving cognitive dysfunction and persistent repetitive behaviors.
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I owe a great debt of gratitude to my collaborators. I thank Dr. Steven Clapcote, Dr. Tatiana Lipina, and Dr. Ina Weiner for guidance with the behavioral measures; Dr. Steven Barger, Ms. Wei Wang, Dr. Glen Baker, Ms. Gail Rauw, and Edward Weiss for their assistance with several biochemical techniques; Dr. Steven Duffy for his help with electrophysiology; and Dr. Albert Wong and his lab for their help with the microarray and human SNP studies. I am also very grateful towards the many members of the Roder lab and the Samuel Lunenfeld Research Institute who have provided help throughout my project. Their advice and support is greatly appreciated.

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<td>5-hydroxytryptamine (serotonin) receptor 2</td>
</tr>
<tr>
<td>A period</td>
<td>Before noise</td>
</tr>
<tr>
<td>ALX-5407</td>
<td>(R)-N-[3-(4’-fluorophenyl)-3(4’-phenylphenoxy)propyl]sarcosine hydrochloride</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Asc-1</td>
<td>Alanine-serine-cysteine transporter 1</td>
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<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>B period</td>
<td>During noise</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>C-terminus</td>
<td>Carboxy terminus</td>
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<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-o-methyltransferase</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>D-s</td>
<td>D-serine</td>
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<td>D1</td>
<td>Dopamine receptor 1</td>
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<td>Dopamine receptor 2</td>
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<tr>
<td>DAO</td>
<td>D-amino acid oxidase</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double-distilled water</td>
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<tr>
<td>DISC1</td>
<td>Disrupted-in-schizophrenia 1</td>
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<tr>
<td>DMP</td>
<td>Delayed-matching-to-place</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNaseI</td>
<td>Deoxyribonuclease I</td>
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<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
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DTT  Dithiothreitol
Dysbindin  Dystrobrevin binding protein 1
E  East
EDTA  Ethylenediaminetetraacetic acid
ENU  N-nitroso-N-ethylurea
ERK  Extracellular signal-regulated kinase
FOs  Familiar objects
GABA  Gamma-aminobutyric acid
GluR1  \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit 2
GluR2  \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit 2
GlyT-1  Glycine transporter 1
GlyT-2  Glycine transporter 2
Grin1  Glutamate receptor, ionotropic, \(N\)-methyl-D-aspartate 1
GRIP  Glutamate-receptor-interacting-protein
GRM3  Metabotropic glutamate receptor 3
H2O2  Hydrogen peroxide
HCl  Hydrochloric acid
HDAC  Histone deacetylase
HpaII  Haemophilus parainfluenzae II
HPLC  High-performance liquid chromatography
HpyCH4V  Helicobacter pylori CH4V
IgG  Immunoglobulin G
ITI  Inter-trial interval
KAT-1  Kynurenine aminotransferase-1
KCl  Potassium chloride
KO  Knockout
KYNA  Kynurenic acid
L-701,324  7-chloro-4-hydroxy-3(3-phenoxy)phenylquinoline-2-(H)-one
L-s  L-serine
<table>
<thead>
<tr>
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<tr>
<td>LI</td>
<td>Latent inhibition</td>
</tr>
<tr>
<td>LSD</td>
<td>Fisher’s least significant difference</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptors</td>
</tr>
<tr>
<td>MlsI</td>
<td>Micrococcus luteus I</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>N</td>
<td>North</td>
</tr>
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<td>Generation</td>
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<td>Sodium chloride</td>
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<td>Sodium hydroxide</td>
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<td>North-east</td>
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<tr>
<td>(NH₄)₂SO₄</td>
<td>Ammonium sulfate</td>
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<td>NMD</td>
<td>Nonsense-mediated mRNA decay</td>
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<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<td>P</td>
<td>Startle-pulse-alone</td>
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<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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</table>
PE  Preexposed
PICK1  Protein-interacting with kinase C
PIP2  Phosphatidylinositol (4,5)-bisphosphate
PKC  Protein kinase C
PLP  Pyridoxal L-phosphate
PPI  Prepulse inhibition
PRODH  Proline dehydrogenase
*R. gracilis*  Rhodotorula gracilis
RGS4  Regulator of G-protein signaling 4
RM  Repeated measures
RNA  Ribonucleic acid
rRNA  Ribosomal ribonucleic acid
RT-PCR  Reverse transcription-polymerase chain reaction
S  South
S(#)  Session
SAM  S-adenosylmethionine
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE  South-east
SEM  Standard error of the mean
SNARE  N-ethyl-maleimide-sensitive factor attachment protein receptor
SNAT  Small neutral amino acid transporter, System A-family
Srr  Serine racemase
SW  South-west
*Taq*  *Thermus aquaticus* polymerase
TBS  Tris buffered saline
TBS-T  Tris buffered saline with 0.1% Tween-20
TGCE  Temperature gradient capillary electrophoresis
TRIS  Tris(hydroxymethyl)aminomethane
US  Unconditioned stimulus
UV  Ultraviolet
W  West
The following publications were completed based on the work presented in this thesis:

Lipina T, **Labrie V**, Weiner I, Roder JC. Modulators of the glycine site on NMDA receptors, D-serine and ALX-5407, display similar beneficial effects to clozapine in mouse models of schizophrenia. Psychopharmacol. 2005;179:54-67.

**Labrie V**, Lipina T, Roder JC. Mice with reduced NMDA receptor glycine affinity model some of the negative and cognitive symptoms of schizophrenia. Psychopharmacol. 2008;200:217-30.


Published as:

Schizophrenia

Schizophrenia is a chronic and severely debilitating psychiatric disorder affecting nearly 1% of the population worldwide. It is characterized by positive symptoms that include hallucinations, delusions, and thought disorder, by negative symptoms comprised of affective flattening and social isolation, and by profound cognitive deficits in attention, learning, memory, and behavioral flexibility\(^1,2\). Symptoms of schizophrenia typically emerge during adolescence or early adulthood and while positive symptoms often fluctuate, negative and cognitive symptoms are more enduring, causing great disability and deterioration in the quality of life of patients\(^3,4\). Current antipsychotic treatments for schizophrenia show success in reducing the severity of positive symptoms, but have limited efficacy in ameliorating negative and cognitive deficits\(^1\). Furthermore, antipsychotic regimes are often poorly tolerated, leading to poor compliance and symptomatic relapse\(^2\). In order to develop effective therapies, much effort has been made to further understand the molecular alterations involved in the pathophysiology of schizophrenia.

Abnormalities in several neurotransmitter systems have been implicated in the pathophysiological processes underlying schizophrenia. The predominant theory has been the dopamine hypothesis, which postulates that schizophrenic symptoms arise from excessive dopaminergic transmission, particularly in the striatum, and the presence of dopaminergic deficits in prefrontal brain regions\(^5\). This theory was based on the observation that blockade of D2 receptors is a mechanism of action for antipsychotics\(^6,7\), and the ability of dopamine-releasing stimulants, such as amphetamine, to induce psychosis\(^8\). Amphetamine elicits only the positive symptoms of schizophrenia, consistent with the greater efficacy of antipsychotics in relieving the positive symptoms rather than the negative symptoms, cognitive
impairments, and cortical atrophy seen in schizophrenia patients. In addition to dopaminergic abnormalities, NMDA receptor (NMDAR) hypofunction has been proposed to be involved in schizophrenia. This theory originated from studies demonstrating that non-competitive NMDAR antagonists like phencyclidine (PCP) reliably and immediately induce a syndrome similar to schizophrenia in healthy individuals, and exacerbate symptoms in schizophrenia patients\textsuperscript{9, 10}. Serum concentrations of PCP that are able to produce psychiatric symptoms correspond to the level that blocks NMDARs\textsuperscript{9}. Moreover, NMDAR inhibitors generate the negative and cognitive disturbances as well as the psychotic symptoms characteristic of the disorder\textsuperscript{9, 10}.

Since these initial observations, several lines of evidence have supported a role for aberrant NMDAR-mediated neurotransmission in schizophrenia pathogenesis\textsuperscript{11, 12}, and the glutamatergic system in schizophrenia is considered to be part of a larger complex framework involving the interaction of multiple neurotransmitters (i.e. dopamine, GABA) and risk genes. Subanesthetic doses of a non-competitive NMDAR antagonist increases dopamine release in the ventral striatum\textsuperscript{13} and enhance amphetamine-induced striatal dopamine release to an extent that mimics the exaggerated responses seen in schizophrenic subjects\textsuperscript{14}. This suggests that psychosis and changes in dopamine release may be the consequence of a defect in the regulatory corticolimbic glutamatergic neuronal pathway\textsuperscript{15}. Chronic exposure to NMDAR inhibitors in rodents and primates also lowers dopamine levels in the prefrontal cortex and affects dopamine receptor binding\textsuperscript{16, 17}, consistent with findings in patients with schizophrenia\textsuperscript{5, 18}. In addition, antagonists of the NMDAR disrupt activity in the prefrontal cortex, affecting the efficiency of neuronal firing and synchronization\textsuperscript{19, 20}, which may contribute to disturbances in cortical processing and cognitive function observed
in schizophrenia. Furthermore, genetic association studies have identified a number of susceptibility genes that influence NMDAR function\textsuperscript{1, 21}. In drug-naïve schizophrenia patients, decreased \textit{in vivo} hippocampal NMDAR binding and reduced plasma levels of endogenous NMDAR agonists have been reported\textsuperscript{22-24}. Postmortem studies have found numerous alterations in NMDAR receptor binding, transcript levels, and subunit protein expression in the cortex, hippocampus, and thalamus of schizophrenic individuals\textsuperscript{25}. Reductions in parvalbumin-immunoreactive cells (GABAergic interneurons) and diminished expression of GAD67, the GABA synthesis enzyme, are also frequently observed in the postmortem hippocampus and prefrontal cortex\textsuperscript{26, 27}. Administration of NMDAR antagonists can replicate the loss of parvalbumin and GAD67\textsuperscript{28, 29}, alter GABA-mediated inhibitory control of cortical neurons\textsuperscript{30}, and disrupt the development of GABAergic neurons\textsuperscript{31}. Thus, NMDA hypofunction could contribute to the abnormalities in several genes and neurotransmitter systems implicated in the biological mechanism underlying schizophrenia.

The indication of aberrant NMDAR function in schizophrenia pathogenesis prompts a need to further understand how NMDAR hypofunction may arise in this disease, and predicts that this system could be useful for the development of novel therapeutics. Recent genetic, clinical, postmortem, pharmacological, and animal studies indicate that the NMDAR glycine site and its modulatory enzymes may be involved in the pathophysiology and treatment of schizophrenia\textsuperscript{11, 12}.

\textbf{The NMDAR: structure and regulation}

NMDARs in the central nervous system (CNS) are heteromeric protein complexes composed of at least one NR1 subunit together with different combinations of NR2 and/or
NR3 subunits\textsuperscript{32}. Alternative splicing of the \textit{Grin1} gene produces eight NR1 isoforms and by associating with different constellations of NR2 (NR2A-D) and NR3 subunits (NR3A and NR3B), form a multitude of different NMDAR receptors with distinct biophysical properties\textsuperscript{33} and specific patterns of expression during development and in the mature mammalian CNS\textsuperscript{34}. NMDAR complexity is further enhanced through post-translational modifications, such as phosphorylation, glycosylation, and ubiquitination, affecting cellular localization and function of the receptor\textsuperscript{35, 36}.

At resting membrane potential, the pore of the NMDAR channel is blocked by magnesium, and this block can be removed via \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor-mediated membrane depolarization\textsuperscript{36}. The NMDAR also contains a glutamate recognition site on the NR2 subunit, and a glycine or D-serine modulatory site on the NR1 subunit (Figure 1.1)\textsuperscript{37, 38}. The glycine/D-serine site on the NMDAR must be occupied for glutamate to activate the receptor\textsuperscript{38}. The unique property of being both voltage-dependent and ligand-gated gives the NMDAR the ability to act as a coincidence detector for presynaptic activity (glutamate release) and post-synaptic activity (adequate depolarization of the post-synaptic membrane). Once activated, the NMDAR channel permits the influx of calcium, which stimulates intracellular signaling cascades that can subsequently affect synaptic plasticity and gene transcription\textsuperscript{39}. Induction of NMDAR-dependent forms of synaptic plasticity, such as long-term potentiation (LTP), is thought to underlie many types of learning and memory formation\textsuperscript{40}.

NMDARs are present throughout the brain, and are principally neuronal, though they can also be expressed on astrocytes\textsuperscript{41, 42}. Beyond the glutamate and glycine binding site, they contain several regulatory sites sensitive to polyamines, Zn\textsuperscript{2+}, protons, and glutathione\textsuperscript{32, 36}. 
The numerous influences that converge on the NMDAR highlight the importance of these receptors in diverse brain functions. Additionally, the NMDAR can also be modulated by several artificially-derived inhibitory compounds, including PCP, MK-801, and ketamine, which are high affinity open channel blockers, and L-701,324 which selectively antagonizes the NMDAR glycine site (Figure 1.1)\(^{32,36,43}\).

**Figure 1.1. The NMDA receptor.** Activation of the receptor requires membrane depolarization in order to remove the magnesium block, along with concurrent binding of glycine or D-serine to the NR1 subunit and glutamate to the NR2 subunit.

**Endogenous modulators of the NMDAR glycine site**

In NMDAR complexes containing NR1 and NR2 subunits, D-serine and glycine both have excitatory effects, with D-serine being up to three times more potent than glycine\(^{44}\).
NMDARs with NR2/NR1 subunits have been implicated in numerous physiological processes, including synaptic plasticity and development, as well as in several pathological conditions, such as neurodegenerative and psychiatric diseases\textsuperscript{32}. In receptors composed of NR1 and NR3 subunits, glycine continues to act as an activator, while D-serine exerts weak partial agonistic effects\textsuperscript{45, 46}. To date, the role of NR3/NR1 heteromers in the adult brain is unclear and they display a peculiar resistance to psychotomimetic NMDAR antagonists\textsuperscript{45, 46}, thus, bringing their relevance to the induction of psychotic syndromes into question.

In addition to enabling NMDAR activation, endogenous glycine site agonists have a role in neuromodulation. Binding to the glycine site allosterically influences the NMDAR to enhance the affinity and efficacy of glutamate\textsuperscript{47}, delays receptor desensitization to increase the duration and frequency of the open channel state\textsuperscript{48}, and promotes NMDAR turnover through priming of the receptor for internalization\textsuperscript{49}.

Examination of the crystal structure of the NR1 binding core provided further insight on the modes of interaction and selectivity of glycine site agonists. D-serine binds more tightly to the receptor than glycine, due to its ability to make 3 additional hydrogen bonds and displace a water molecule within the binding pocket\textsuperscript{50}. The binding of the D-enantiomer to NR1 is selective, as L-serine contains a hydroxyl group that interacts unfavorably with a phenyl ring in the binding site\textsuperscript{50}.

Whether the glycine site on NMDARs is saturated by glycine or D-serine at physiological conditions remains somewhat controversial, though most evidence indicates differing levels of occupancy in various brain regions. Elevations in glycine and/or D-serine concentrations evokes NMDAR responses in the prefrontal cortex, visual cortex, neocortex, hippocampus, thalamus, and brainstem, signifying that the NMDAR glycine may not be fully
saturated at synapses in these brain regions\textsuperscript{51-56}. On the other hand, the glycine site at cerebellar synapses may be saturated, as the addition of glycine or D-serine does not further augment NMDAR-mediated synaptic currents in this area\textsuperscript{57}. Incomplete saturation of the glycine site in several brain regions suggests that agonists of the glycine site are capable of regulating NMDAR-mediated neurotransmission.

\textbf{Glycine}

Although glycine is an abundant amino acid found throughout the brain, its synaptic concentrations are tightly regulated by glycine transporters. At NMDAR-expressing synapses, extracellular glycine concentrations are primarily derived from astroglial cells and clearance is mediated by glycine transporter 1 (GlyT-1)\textsuperscript{58,59}. GlyT-1s are closely associated to NMDARs\textsuperscript{60} and there at least 6 glial and neuronal subtypes (GlyT-1\textsubscript{a-f})\textsuperscript{61,62}. GlyT-1 effectively maintains low, subsaturating levels of glycine, as GlyT-1 blockers like ALX-5407 are capable of enhancing NMDAR activity\textsuperscript{51,63,64}. Spillover from glycinergic neurons also contributes a small amount of glycine to NMDARs\textsuperscript{65}; although distant diffusion from glycinergic neurons is limited by the high affinity glycine transporter 2 (GlyT-2) that is responsible for glycine reuptake near strychnine-sensitive glycine A receptors\textsuperscript{66}. In addition, System A-family transporters (SNAT) on astrocytes and neurons transport a range of small neutral amino acids including glycine, and by modulating glycine uptake and release may also contribute to the dynamic regulation of extracellular glycine\textsuperscript{67-69}.

Biosynthesis of glycine occurs through the conversion of L-serine to glycine by the enzyme serine hydroxymethyltransferase\textsuperscript{70}. This enzyme is also capable of functioning in a reverse direction, thereby eliminating glycine\textsuperscript{70}. Additionally, the glycine cleavage system in
astrocytes efficiently degrades glycine, and generates the concentration gradient between the cytosol and extracellular space that allows glycine transporters to remove glycine from the synaptic cleft\textsuperscript{71, 72}.

**D-serine**

The discovery of D-serine in the brain revolutionized the long-standing belief that only L-isomers of amino acids existed in mammalian tissues\textsuperscript{73}. D-serine was found to be a highly selective endogenous activator of the NMDAR glycine site\textsuperscript{74-76}. The origin of D-serine in mammals was puzzling until the identification of the enzyme serine racemase (Srr), which directly converts L-serine to D-serine\textsuperscript{77} in the presence of the co-factors pyridoxal 5’phosphate, magnesium, and ATP\textsuperscript{78}. Srr is also able to convert D-serine into L-serine with a much lower affinity, and possesses α,β-elimination activity capable of transforming L- or D-serine into pyruvate and ammonia\textsuperscript{78, 79}. Like D-serine, Srr is present in both astrocytes and neurons of the brain\textsuperscript{77, 80-83}, with a regional distribution that closely resembles that of NMDARs\textsuperscript{84}. Though enriched in the brain, Srr protein has also been detected in the murine liver and the human liver, kidney, and heart\textsuperscript{77, 85}. Similarly, D-serine levels are much higher in the CNS than in peripheral tissues\textsuperscript{86}. In the adult human and rodent brain, D-serine and Srr are predominantly localized to the forebrain, with high levels in the cerebral cortex and hippocampus, and minimal levels in the cerebellum and brainstem\textsuperscript{85, 87}. The low D-serine concentrations in caudal brain areas coincide with the emergence of D-amino acid oxidase (DAO), the D-serine catabolic enzyme\textsuperscript{88, 89}. Prior to the appearance of DAO, D-serine levels are relatively high in caudal regions of the developing brain, where D-serine released from Bergman glia mediates NMDAR-dependent neuronal migration in the cerebellum\textsuperscript{90}. 
DAO is highly selective for D-serine degradation at physiological pH, where it catalyzes the oxidative deamination of D-serine to produce an α-keto acid, ammonia, and hydrogen peroxide\textsuperscript{91, 92}. The brain distribution of DAO is inversely related to that of endogenous D-serine concentrations, with the highest levels of DAO in astrocytes, Golgi-Bergmann glia, and tanycytes of the hindbrain and cerebellum\textsuperscript{93}. Lower levels of DAO have been detected in the neurons of the prefrontal cortex, hippocampus, and substantia nigra\textsuperscript{93, 94}. In the periphery, DAO is most highly expressed in the kidneys and liver\textsuperscript{95}.

Modulating DAO function is G72 (also known as LG72 or DAO activator), a gene unique to primates\textsuperscript{96}. Initially, G72 was reported to be an activator of DAO\textsuperscript{96}; however a recent study indicates that G72 may instead repress DAO activity\textsuperscript{97}. The function of G72 remains controversial, as in mammalian cell lines and rat primary hippocampal neurons, G72 was described to have an alternate role, acting as a mitochondrial protein that promoted mitochondrial fragmentation and dendritic arborization\textsuperscript{98}. Though G72 mRNA has been detected in the human dorsolateral prefrontal cortex\textsuperscript{99}, difficulties in detecting native G72 protein have been noted\textsuperscript{100}.

Recent studies have begun to elucidate the regulatory pathway of D-serine and serine racemase (Figure 1.2). Activation of astrocytic AMPA receptors stimulates D-serine release, an effect that is dependant on the rise of intracellular calcium and the soluble N-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE)\textsuperscript{101}. Calcium has been shown to bind and activate Srr\textsuperscript{102}, indicating that Srr activity is regulated by neuronal depolarization and the related calcium entry. The binding of glutamate-receptor-interacting-protein (GRIP) to Srr enhances the formation of D-serine\textsuperscript{103}. It has been proposed that either GRIP disassociates from phosphorylated AMPA receptors and activates Srr in the cytosol, or GRIP
mediates translocation of Srr to the proximity of AMPA receptors for the formation of a ternary complex between the GluR2 subunit of the AMPA receptor, SRR, and GRIP\textsuperscript{104}. Activation of metabotropic glutamate receptors (mGluR5) on astrocytes have also recently been shown to enhance D-serine production and serine racemase activity by diminishing phosphatidylinositol (4,5)-bisphosphate (PIP2) inhibition of serine racemase\textsuperscript{105}. Additionally, protein-interacting with kinase C (PICK1) has been shown to bind to the C-terminus of Srr\textsuperscript{106}, possibly to directly modulate its activity. Alternatively or in addition, PICK1 may escort protein kinase C (PKC) to Srr, leading Srr phosphorylation and altered Srr activity\textsuperscript{106}. Following synthesis, D-serine undergoes vesicular storage and release from astrocytes\textsuperscript{80, 101, 107} or is released through a nonvesicular pathway from neurons\textsuperscript{83} and possibly astrocytes\textsuperscript{108}. As mentioned earlier, intracellular levels of D-serine are regulated by Srr and DAO; however clearance of D-serine from the synaptic space is assured by various sodium-dependent and sodium-independent transporters expressed on neurons and glia\textsuperscript{108-111}. Among the transporters, the alanine-serine-cysteine transporter 1 (Asc-1) mediates the majority of D-serine reuptake in the brain\textsuperscript{112}. Asc-1 is located on the presynaptic terminal, dendrites, and cell body of neurons\textsuperscript{110} and is the only transporter that exhibits a high D-serine affinity\textsuperscript{112}. 
Figure 1.2. Schematic of putative D-serine pathway. Serine racemase (Srr) synthesizes D-serine (▲) from L-serine (▼), while D-amino acid oxidase (DAO) is responsible for D-serine degradation. Stimulation of AMPA receptors (AMPAR) and association of glutamate-receptor-interacting-protein (GRIP) with Srr increases the production of D-serine. Protein-interacting with kinase C (PICK1) also binds to Srr, possibly to directly regulate its activity or to bring protein kinase C into the vicinity of Srr. D-serine is released via vesicular and non-vesicular pathways into the synapse, where it then binds to the NMDA receptor (NMDAR) glycine site. Sodium-dependent and -independent transporters, such as Asc-1, mediate D-serine clearance from the extracellular space.
The predominant physiological co-agonist of the NMDAR glycine site

Growing evidence indicates that D-serine, rather than glycine, is the dominant endogenous ligand for the glycine site of most NMDARs. Depletion of D-serine by treatment with DAO has been shown to attenuate NMDAR activity, as measured by biochemical and electrophysiological approaches in cerebellar slices, hippocampal slices, hippocampal cell cultures, and retina preparations\(^74,113,114\). Moreover, in hypothalamic slices, NMDAR currents are substantially reduced following elimination of D-serine by DAO, while a loss of glycine by a glycine oxidase enzyme does not produce an effect\(^115\). Similarly, removal of D-serine with a recombinant D-serine deaminase enzyme suppressed NMDAR-mediated light-evoked responses in retinal cells and NMDAR-induced neurotoxicity in organotypic hippocampal slices\(^75,114\). In a senescence-accelerated mouse strain, deficient NMDAR-dependent LTP in the hippocampus was associated with a diminished production of D-serine, but not lowered levels of glycine\(^116\). The effects of diminished NMDAR-mediated neurotransmission in these experiments could be fully reversed by the application of exogenous D-serine\(^74,113-116\). Together, these experiments favor D-serine as the predominant physiological co-agonist for the NMDAR glycine site.

Increases in D-serine are also capable of further enhancing NMDAR signaling. This has been demonstrated several in vitro studies examining NMDAR-evoked excitatory responses in the prefrontal cortex, hippocampus, striatum, and in hippocampal cultures\(^51,113,117,118\). In mice that lack DAO activity, the resulting elevation in D-serine potentiates NMDAR-mediated currents in spinal cord neurons\(^119\). Mice that lack the neuronal transporter Asc-1 also display NMDAR-dependent hyperexcitability\(^120\), presumably from the elevations in extracellular D-serine. Finally, exogenous administration of D-serine has been shown to
elevate hippocampal responses \textit{in vivo}, as measured by changes in relative cerebral blood volume in a functional magnetic resonance imaging study\textsuperscript{54}.

Thus, these findings demonstrate a capacity for D-serine and its modulatory enzymes to dynamically regulate NMDAR activity, and disturbances in this pathway could conceivably contribute to psychopathologies associated with abnormal NMDAR-mediated neurotransmission.

**Genetic studies: G72, DAO, and Srr**

Archival family, twin, and adoption studies indicate that schizophrenia is highly heritable, but no single gene exhibits a strong effect. Instead, accumulating evidence indicates that schizophrenia has a heterogeneous etiology involving a complex interplay of multiple genes, epigenetics, and environmental factors\textsuperscript{1,21}. Several of the genes associated with schizophrenia risk are modulators of NMDAR glycine site activation. In particular, genes involved in D-serine catabolism and synthesis have been identified.

\textit{G72} was initially identified by Chumakov et al.\textsuperscript{96}, who examined markers within a 5-Mb segment from chromosome 13q33, a region that had previously been linked to schizophrenia in earlier linkage analyses\textsuperscript{121}. A significant association between \textit{G72} and schizophrenia was identified in French Canadian and Russian populations\textsuperscript{96}. In a yeast two-hybrid screen, \textit{G72} strongly associated with DAO, and in vitro assays confirmed a regulatory effect of \textit{G72} on DAO activity\textsuperscript{96,97}. The positive association of \textit{G72} with schizophrenia susceptibility has since been replicated in numerous studies\textsuperscript{99,122-125} and continues to be significant following meta-analyses\textsuperscript{126}. Additionally, \textit{G72} is one of the best supported locus for bipolar disorder\textsuperscript{123,124,127,128}. Furthermore, some evidence indicates an association with
major depression and panic disorder\textsuperscript{129, 130}, with one large study of 2831 individuals suggesting that \textit{G72} may influence predisposition to episodes of mood disorder across traditional bipolar and schizophrenia categories\textsuperscript{128}. Despite the number of positive associations, these studies have demonstrated considerable allelic heterogeneity, with few studies reporting association with the same allele at a SNP marker. The limited allelic compatibility along with the difficulties in identifying endogenous \textit{G72} protein\textsuperscript{100} have made it difficult to understand how \textit{G72} is dysregulated in schizophrenia and other psychiatric illnesses. However, Korostishevsky et al.\textsuperscript{99} did amplify \textit{G72} mRNA showing that it is overexpressed in the dorsolateral prefrontal cortex of postmortem schizophrenia patients.

Interestingly, recent genetic classification and fMRI investigations in healthy and schizophrenic populations report that genetic variation in the \textit{G72} gene may influence cognitive function\textsuperscript{125, 131-133}. Carriers of \textit{G72} risk variants differed in their performance during tests of working memory, verbal initiation, attention, and semantic fluency, and displayed differential recruitment of brain regions relevant to cognitive ability, including the hippocampal complex and prefrontal cortex\textsuperscript{125, 131-133}. By modulating DAO activity, \textit{G72} could contribute to the regulation of NMDAR-mediated cognitive function in the healthy brain and impact an array of diseases characterized by cognitive impairment.

The Chumakov et al.\textsuperscript{96} study also demonstrated that SNP markers within the \textit{DAO} gene may confer an increased vulnerability to schizophrenia, as surveyed in a French Canadian population. This has been independently replicated in a number of subsequent genetic investigations in German, Han Chinese, Irish, American schizophrenia samples\textsuperscript{123, 134-136}. Furthermore, some studies have indicated epistasis between \textit{DAO} and \textit{G72}, where the combined effect of polymorphisms in these genes results in a greater risk of schizophrenia\textsuperscript{96,}
However, negative studies examining DAO have been reported\textsuperscript{24, 122, 137}, and the evidence for an association between DAO and schizophrenia susceptibility is not as prevalent as that for G72. Similar to G72, functional (or protective) variants in DAO have yet to be identified.

Preliminary studies have indicated that genetic variation in the Srr gene could contribute to schizophrenia. Investigations examining variants in the promoter and 5’-terminus of Srr have demonstrated significant associations with schizophrenia\textsuperscript{138-140}, contrary to polymorphisms in the central and 3’ region of Srr\textsuperscript{140, 141}. Consequently, it is possible that the 5’ end of Srr is of importance in mediating abnormal Srr function in schizophrenia. Accordingly, a significantly associated variant was found to induce a 60% reduction in Srr promoter function\textsuperscript{139}. Furthermore, all Srr markers demonstrating a positive association are in close proximity to exon 1b, the major Srr isoform in the brain\textsuperscript{24}, suggesting a potential modulation of Srr transcription in the brain.

\textit{PICK1}, an interacting partner of Srr, has also been described to confer susceptibility to schizophrenia, particularly to the disorganized subtype\textsuperscript{106, 142}. The \textit{PICK1} gene is found on chromosome 22q13.1, which a genetic locus that has been often linked to schizophrenia\textsuperscript{121}. PICK1 is a scaffolding protein that regulates the subcellular localization and surface expression of a number of binding partners\textsuperscript{143}, many of which are relevant to schizophrenia, including glutamate receptors, dopamine transporters, neuregulin, and ErbB tyrosine kinase receptors\textsuperscript{143}. PICK1 has also been demonstrated to have an important role in NMDAR-dependent forms of synaptic plasticity\textsuperscript{144}.

In addition, several genes that affect glutamatergic neurotransmission have been identified as promising candidate risk genes for schizophrenia\textsuperscript{11}. These include genes that regulate NMDAR expression (neuregulin 1), glutamate release (dysbindin, GRM3), and
NMDAR activity (calcineurin)\(^{11,12}\). To date, GlyT-1 variants have not been associated with a greater vulnerability to schizophrenia\(^{145}\).

**Evidence for abnormal modulation of the NMDAR glycine site in patients with schizophrenia**

Kynurenic acid (KYNA) is the only known endogenous NMDAR glycine site antagonist\(^{146}\). It also functions as a non-competitive inhibitor of \(\alpha-7\) nicotinic acetylcholine receptors\(^{147}\). Elevations in kynurenic acid have been found in the CSF and postmortem brain of schizophrenia patients\(^{148,149}\). Additionally, increased activity of kynurenine aminotransferase-1 (KAT-1), a synthesis enzyme for KYNA, was reported in schizophrenic individuals (Table 1.1)\(^{150}\).

D-serine levels were found to be reduced in the CSF of drug-naïve patients with schizophrenia\(^{151,152}\). Serum analysis has also indicated diminished D-serine, along with a concomitant elevation in L-serine, suggesting a dysfunction of Srr activity\(^{23}\). Indeed, changes in Srr protein expression have been reported in the postmortem hippocampus and cortex of schizophrenic individuals, with some studies indicating a decrease\(^{152}\), while others an increase (Table 1.1)\(^{94,153}\). Postmortem findings have also demonstrated an elevation in DAO protein in the hippocampus and cerebellum of patients with schizophrenia\(^{94,152}\), as well as an increase in cortical DAO activity (Table 1.1)\(^{150,154}\). Further support for abnormalities in the D-serine pathway in schizophrenia is indicated by reductions in PICK1 mRNA\(^{155}\) and Asc-1 protein\(^{156}\) in the prefrontal cortex. It has been speculated that lower levels of Asc-1 may be a response to diminished D-serine availability\(^{156}\), though further investigation is required. Additionally, postmortem findings will benefit from replication with more brain series and
regions to determine the extent of the abnormal expression and function of NMDAR glycine site modulators in schizophrenia. Use of biopsied olfactory epithelium from living patients\textsuperscript{157} may also reveal molecular changes associated with the NMDAR glycine site, while limiting potential confounds that include the effects of long-term exposure to medications, agonal state, and postmortem interval.

Although conventional antipsychotics do not directly alter D-serine levels, improvement of schizophrenia symptoms are correlated with an elevation in D-serine\textsuperscript{158}. A similar effect has been observed for glycine. In medication-free schizophrenia patients, circulating levels of glycine have been found to be reduced, and glycine availability inversely correlates with the severity of negative symptoms\textsuperscript{159,160}. Treatment efficacy to ameliorate these negative symptoms correlated with a rise in plasma glycine levels\textsuperscript{161}. Together these studies suggest an abnormality in available glycine and/or D-serine in schizophrenia, and predict the potential therapeutic utility of these compounds.
Table 1.1. Changes in the expression of genes affecting the NMDAR glycine site in postmortem schizophrenic brain

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prefrontal Cortex</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
<th>Refs</th>
</tr>
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<tbody>
<tr>
<td>Serine racemase (Srr)</td>
<td>mRNA: NS</td>
<td>mRNA: NS</td>
<td>Protein: ↓</td>
<td>mRNA: NS</td>
<td>94, 150, 152, 153</td>
</tr>
<tr>
<td></td>
<td>Protein: ↑, NS</td>
<td>Protein: ↓, NS</td>
<td>↑</td>
<td>Protein: NS</td>
<td></td>
</tr>
<tr>
<td>D-amino acid oxidase (DAO)</td>
<td>mRNA: NS</td>
<td>mRNA: NS</td>
<td>Protein: ↑</td>
<td>mRNA: ↑, ↑</td>
<td>94, 150, 152, 154</td>
</tr>
<tr>
<td></td>
<td>Protein: ↑, ↑</td>
<td>Protein: t↑</td>
<td>Activity: ↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine-serine-cysteine transporter (Asc-1)</td>
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<td>mRNA: NS</td>
<td>Protein: ↓</td>
<td>mRNA: NS</td>
<td>156</td>
</tr>
<tr>
<td>Glycine transporter 1 (GlyT-1)</td>
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<td>mRNA: NS</td>
<td>Protein: ↓</td>
<td>mRNA: ↓</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Protein: NS</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>System A amino acid transporter 2 (SNAT2)</td>
<td>mRNA: ↓</td>
<td>mRNA: ↓</td>
<td></td>
<td></td>
<td>156</td>
</tr>
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<td>Kynurenine aminotransferase-1 (KAT-1)</td>
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<td>mRNA: NS</td>
<td>Activity: ↑</td>
<td>mRNA: NS</td>
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<td></td>
<td>Activity: NS</td>
<td>Activity: ↑</td>
<td></td>
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</tr>
</tbody>
</table>

NS = no significant change; ↑ = increase; ↓ = decrease; t↑ = trend for increase (near significance)

Pharmacological treatments targeting the NMDAR glycine site

Since NMDAR hypoactivity potentiates schizophrenia-like symptoms and is implicated in the pathophysiology of schizophrenia, it may follow that NMDAR activation could alleviate symptoms of this disorder. The NMDAR glycine site has been proposed as a potential therapeutic target, as increasing its activation offers a safer alternative to elevations in glutamate levels that can promote neurotoxicity. To date, clinical trials have been conducted with the partial agonist D-cycloserine, the full agonists glycine and D-serine, and the GlyT1 inhibitor sarcosine (Table 1.2). Supporting the therapeutic effectiveness of correct modulation of disrupted glutamatergic pathways are clinical reports that activation of mGluR2/3 receptors provides symptomatic improvements in schizophrenia patients without major adverse effects.
An initial study assessing the clinical efficacy of D-cycloserine in conjunction with conventional medications observed a U-shaped dose response curve\textsuperscript{164}, since as a partial agonist D-cycloserine can function as an agonist or antagonist depending on the degree of occupancy at the glycine site\textsuperscript{165}. In this study of only 9 patients with doses escalating every 2 weeks, improvements in negative and cognitive deficits were found at a dose of 50 mg/day\textsuperscript{164}. Although the capacity of D-cycloserine to improve negative symptoms has been replicated in some larger placebo-controlled studies\textsuperscript{166,167}, evidence supporting the effectiveness of D-cycloserine is weak. D-cycloserine was not found to be beneficial as adjunctive treatment in a 6-month placebo-controlled trial\textsuperscript{168}, in a large multicenter 16-week trial with a placebo comparison\textsuperscript{169} nor in a systematic review of the literature\textsuperscript{170}. Amidst all these negative findings, one study shows that D-cycloserine enhances temporal lobe activation in schizophrenia patients during a memory task, and that this response is correlated with a significant decrease in negative symptoms\textsuperscript{171}. However, the overall disparity in findings indicates a limited therapeutic effect of D-cycloserine in the general patient population.

Clinical trials examining the effects of a high dose of glycine (0.8 g/kg/day) administered as adjuvant treatment have demonstrated promising results, particularly in the amelioration of primary negative symptoms in patients with chronic schizophrenia\textsuperscript{172-174}. Some studies have indicated that glycine may also improve cognitive and positive symptoms\textsuperscript{175,176}, although these additional benefits are not supported in a meta-analysis\textsuperscript{170}. In contrast, a large multicenter study found that glycine administration did not ameliorate negative or cognitive symptoms compared to placebo treatment\textsuperscript{169}. The lack of improvement in this trial may be related to the higher percentage of patients treated with second-generation
antipsychotics rather than conventional antipsychotics. Additionally, serum levels of glycine in this trial were lower compared to those in some of the previous positive studies. Though glycine is generally well-tolerated with minimal serious side effects, glycine treatment has been associated with reoccurring gastrointestinal upset\(^{177}\).

In a preliminary study, D-serine (30 mg/kg) in combination with antipsychotic drugs was found to be therapeutically beneficial, as it considerably reduced positive, negative, and cognitive symptoms of schizophrenia\(^{178}\). These ameliorative effects were confirmed in a subsequent clinical trial in which D-serine was added to risperidone or olanzapine, and improvements in positive, negative, cognitive, and depressive symptoms were found in treatment-resistant patients\(^{179}\). However, in patients with an acute exacerbation of psychosis, D-serine did not produce any benefits beyond risperidone monotherapy\(^{180}\). The lack of beneficial effects could be related to differential treatment responses in acutely ill patients compared to treatment-resistant individuals, and indicates that D-serine may be less effective in treating psychotic symptoms. D-serine was well-tolerated and devoid of significant side effects\(^{178-180}\). One concern with D-serine treatments has been renal toxicity, since large doses of D-serine have been found to cause reversible acute tubular necrosis in rats\(^{181}\). The nephrotoxic effects of D-serine were not observed in mice, guinea pigs, rabbits, dogs, and gerbils\(^{182}\) and analysis of kidney function parameters did not reveal any abnormalities in the clinical trials\(^{178-180}\).

As an alternative to directly activating the NMDAR glycine site, clinical investigations also examined sarcosine, an inhibitor of GlyT-1 that effectively raises synaptic levels of glycine\(^{63}\). Sarcosine (2 g/day) cotreatment with conventional medications or risperidone significantly reduced positive, negative, cognitive, and general psychopathology
symptoms in patients with stable chronic schizophrenia and in patients with acute exacerbation of schizophrenia\textsuperscript{180, 183}. Recently, the effectiveness of sarcosine was examined in a drug-free cohort displaying acute psychotic symptoms; the first clinical trial examining a glycine reuptake inhibitor in absence of other medications\textsuperscript{184}. Though symptomatic amelioration was observed, it was limited to a small patient subgroup with no previous antipsychotic exposure and no placebo control was conducted\textsuperscript{184}. Larger studies with placebo or active comparators will be necessary to determine the therapeutic benefits of NMDAR glycine site agonists as first-line antipsychotics.

A peculiarity of NMDAR glycine site agonists is that although ameliorative effects are found in combination with second-generation antipsychotics, such as risperidone and olanzapine, these treatments are ineffective when administered in conjunction with clozapine (Table 1.3)\textsuperscript{185-187}. Clozapine is a commonly used atypical antipsychotic with a broad pharmacological profile that includes affinity for $D_1$- and $D_2$-like dopamine receptors and 5-HT\textsubscript{2} receptors\textsuperscript{1}. Also, clozapine has been shown to increase extracellular levels of glutamate\textsuperscript{188}, potentiate NMDAR-mediated synaptic transmission\textsuperscript{189}, reverse antagonist blockade of NMDAR channels in vivo\textsuperscript{190}, normalize PCP-induced neuronal hyperactivity in the prefrontal cortex\textsuperscript{191}, and attenuate behavioral deficits induced by NMDAR inhibitors\textsuperscript{192, 193}. Thus, clozapine may augment NMDAR activity to an extent that impedes further enhancements with glycine site agonists. Additionally, clozapine may augment the synaptic levels of glycine, by inhibiting glycine reuptake into neurons and glia\textsuperscript{194, 195}.

Overall, clinical trials with glycine site activators have indicated therapeutic potential. However, thus far, studies are generally conducted with a relatively low number of participants (< 30) over a 6 week period (Table 1.2 and 1.3). Consequently, longer studies
investigating a greater number of patients are required to ascertain the benefits of glycine site agonists. Also, since the majority of studies examine treatment-resistant patients with stable chronic symptoms, assessment of these proposed antipsychotics in a more diverse patient population may reveal additional improvements. Regardless, compounds targeting the glycine site may offer a novel and safe alternative for the treatment of schizophrenia.

Enhanced activity of the NMDAR glycine site may also be beneficial for the treatment of other psychiatric disorders, including several anxiety syndromes and drug addiction, by augmenting the extinction of learned behaviors. Extinction is considered to be a form of inhibitory learning, whereby acquired behavioral responses are suppressed following the repetitive exposure to a conditioned stimulus in the absence of a reinforcing (unconditioned) stimulus. Studies of fear extinction in rats found that administration of D-cycloserine accelerated the effects of extinction, and that this acceleration could be blocked by an NMDAR glycine site antagonist. Since fear extinction in rodents is similar to exposure-based psychotherapy in humans, translational research from preclinical to clinical studies was initiated to examine the therapeutic effects of D-cycloserine. The initial study examined individuals with a fear of heights (acrophobia) and found that compared to placebo, D-cycloserine enhanced the effects of virtual reality exposure therapy, resulting in a larger reduction of acrophobic symptoms at 1 week and 3 months following treatment. Subsequent placebo-controlled studies in patients with obsessive-compulsive disorder and social phobia have confirmed the ability of D-cycloserine to augment the effects of exposure therapy. Additionally, extinction of the preference for a cocaine-associated environment was facilitated by D-cycloserine, suggesting that this partial agonist of the NMDAR glycine site aids in extinguishing craving behaviors related to drug addiction. From
these studies with D-cycloserine, it is not clear whether fear extinction and exposure therapy are facilitated by the augmentation of NMDAR responses during extinction or by the reduction of NMDAR activity during the memory consolidation process.

**Table 1.2. Modulators of the NMDAR glycine site in clinical trials with schizophrenia patients**

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Design</th>
<th>Daily dose</th>
<th>Sample size</th>
<th>Treatment duration</th>
<th>Patient sample</th>
<th>Antipsychotics</th>
<th>Therapeutic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D-cycloserine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cascella et al. 266</td>
<td>open label</td>
<td>250 mg</td>
<td>7</td>
<td>6 weeks</td>
<td>chronic</td>
<td>typical</td>
<td>↓: +, −, gen psychopath</td>
</tr>
<tr>
<td>Goff et al. 164</td>
<td>single blind</td>
<td>5-250 mg</td>
<td>9</td>
<td>10 weeks (2 weeks/dose)</td>
<td>chronic</td>
<td>typical</td>
<td>↑: − at 50 mg</td>
</tr>
<tr>
<td>Rosse et al. 267</td>
<td>double-blind, placebo-controlled</td>
<td>10, 30 mg</td>
<td>13</td>
<td>4 weeks</td>
<td>chronic</td>
<td>molindone</td>
<td>NS</td>
</tr>
<tr>
<td>van Berckel et al. 268</td>
<td>single blind</td>
<td>15-250 mg</td>
<td>7</td>
<td>24 days (4 days/dose) 6 weeks</td>
<td>not specified</td>
<td>drug-free</td>
<td>↑: − at 100 mg</td>
</tr>
<tr>
<td>Heresco-Levy et al. 269</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>9</td>
<td>chronic, treatment-resistant PDS</td>
<td>↑: −</td>
<td></td>
<td>↑: −</td>
</tr>
<tr>
<td>Goff et al. 167</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>38</td>
<td>8 weeks</td>
<td>chronic, prominent negative symptoms</td>
<td>↑: −</td>
<td></td>
</tr>
<tr>
<td>van Berckel et al. 266</td>
<td>double-blind, placebo-controlled</td>
<td>100 mg</td>
<td>25</td>
<td>8 weeks</td>
<td>chronic, prominent negative symptoms treatment-resistant</td>
<td>↑: −</td>
<td></td>
</tr>
<tr>
<td>Heresco-Levy et al. 269</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>16</td>
<td>6 weeks</td>
<td>typical</td>
<td></td>
<td>↑: −, gen psychopath</td>
</tr>
<tr>
<td>Duncan et al. 213</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>22</td>
<td>4 weeks</td>
<td>typical</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Evins et al. 212</td>
<td>single blind</td>
<td>5-250 mg</td>
<td>10</td>
<td>10 weeks (2 weeks/dose) 6 months</td>
<td>risperidone</td>
<td>↑: −</td>
<td></td>
</tr>
<tr>
<td>Goff et al. 168</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>26</td>
<td>chronic, prominent negative symptoms, PDS primary deficit syndrome</td>
<td>typical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buchanan et al. 169</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>133</td>
<td>16 weeks</td>
<td>typical/ataypical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goff et al. 213</td>
<td>double-blind, placebo-controlled</td>
<td>50 mg</td>
<td>33</td>
<td>8 weeks</td>
<td>typical/ataypical</td>
<td>↑: −, cognitive</td>
<td></td>
</tr>
</tbody>
</table>

**Glycine**

<p>| Waziri 214       | open label, naturalistic        | 5-25 g     | 11          | 8-9 months        | chronic        | drug-free      | ↑: −: 4 patients |
| Rosse et al. 215 | open label                      | 10.8 g     | 6           | 4 days-8 weeks    | chronic        | typical        | ↑: −: 2 patients |
| Costa et al. 216  | open label                      | 15 g       | 6           | 5 weeks           | treatment-resistant | typical        | ↑: −: 2 patients |
| Javitt et al. 172 | double-blind, placebo-controlled| 30 g       | 14          | 8 weeks           | chronic        | typical        | ↑: −              |
| Leiderman et al. 217| open label                      | 60 g       | 5           | 8 weeks           | chronic        | typical/ataypical | ↑: −              |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Dose/Route</th>
<th>Duration</th>
<th>Condition</th>
<th>Outcome</th>
<th>Methodology</th>
<th>Patients</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heresco-Levy et al.</td>
<td>double-blind, placebo-controlled</td>
<td>60 g</td>
<td>11</td>
<td>6 weeks chronic, treatment-resistant</td>
<td>typical/atypical</td>
<td>↑: −, cognitive, gen psychopath</td>
<td>↑: 175</td>
<td></td>
</tr>
<tr>
<td>Javitt et al.</td>
<td>double-blind, placebo-controlled</td>
<td>60 g</td>
<td>19</td>
<td>6 weeks treatment-resistant</td>
<td>typical/atypical</td>
<td>↑: −, cognitive</td>
<td>↑: 173</td>
<td></td>
</tr>
<tr>
<td>Heresco-Levy et al.</td>
<td>double-blind, placebo-controlled</td>
<td>60 g</td>
<td>12</td>
<td>6 weeks chronic</td>
<td>typical/atypical</td>
<td>↑: −, cognitive</td>
<td>↑: 176</td>
<td></td>
</tr>
<tr>
<td>Buchanan et al.</td>
<td>double-blind, placebo-controlled</td>
<td>60 g</td>
<td>14</td>
<td>6 weeks chronic, treatment-resistant</td>
<td>olanzapine/risperidone</td>
<td>↑: +, −, cognitive</td>
<td>↑: 180</td>
<td></td>
</tr>
<tr>
<td>Heresco-Levy et al.</td>
<td>double-blind, placebo-controlled</td>
<td>30 mg/kg</td>
<td>28</td>
<td>6 weeks prominent negative symptoms, PDS, treatment-resistant acute illness</td>
<td>typical/atypical</td>
<td>↑: +, −, cognitive</td>
<td>↑: 178</td>
<td></td>
</tr>
<tr>
<td>D-serine</td>
<td>double-blind, placebo-controlled</td>
<td>30 mg/kg</td>
<td>28</td>
<td>6 weeks prominent negative symptoms</td>
<td>typical/atypical</td>
<td>↑: +, −, cognitive</td>
<td>↑: 179</td>
<td></td>
</tr>
<tr>
<td>Lane et al.</td>
<td>double-blind, placebo-controlled</td>
<td>2 g</td>
<td>57</td>
<td>6 weeks treatment-resistant</td>
<td>risperidone</td>
<td>NS</td>
<td>↑: 183</td>
<td></td>
</tr>
<tr>
<td>Heresco-Levy et al.</td>
<td>double-blind, placebo-controlled</td>
<td>30 mg/kg</td>
<td>38</td>
<td>6 weeks treatment-resistant</td>
<td>risperidone/olanzapine</td>
<td>↑: +, −, cognitive</td>
<td>↑: 184</td>
<td></td>
</tr>
<tr>
<td>Sarcosine</td>
<td>double-blind, placebo-controlled</td>
<td>2 g</td>
<td>36</td>
<td>6 weeks chronic</td>
<td>typical/risperidone/drug-free</td>
<td>↑: all</td>
<td>↑: 184</td>
<td></td>
</tr>
<tr>
<td>Lane et al.</td>
<td>double-blind, placebo-controlled</td>
<td>2 g</td>
<td>57</td>
<td>6 weeks acute illness</td>
<td>risperidone</td>
<td>↑: −, cognitive, gen psychopath, gen psychopathology</td>
<td>↑: 184</td>
<td></td>
</tr>
<tr>
<td>Lane et al.</td>
<td>double-blind</td>
<td>1, 2 g</td>
<td>16</td>
<td>6 weeks acute illness</td>
<td>drug-free/drug-naive</td>
<td>↑: −, at 2 g in drug naive patients</td>
<td>↑: 184</td>
<td></td>
</tr>
</tbody>
</table>

↑ = improvement; ↓ = exacerbation; NS = no significant change
Abbreviations: +, positive; −, negative; gen psychopath, general psychopathology; all, +/−/cognitive/general psychopathology; PDS, primary deficit syndrome
†Number of patients that completed the trial
Table 1.3. Clinical trials with modulators of the NMDAR glycine site added to clozapine treatment

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Design</th>
<th>Daily dose</th>
<th>Sample size†</th>
<th>Duration</th>
<th>Patient sample</th>
<th>Therapeutic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D-cycloserine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goff et al.</td>
<td>single blind</td>
<td>5-250 mg</td>
<td>10</td>
<td>10 weeks (2 weeks/dose)</td>
<td>primary deficit syndrome</td>
<td>↓: − at 50 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 mg</td>
<td>11</td>
<td>6 weeks</td>
<td>prominent negative symptoms</td>
<td>−</td>
</tr>
<tr>
<td>Goff et al.</td>
<td>double-blind, placebo-controlled</td>
<td>30 g</td>
<td>19</td>
<td>12 weeks</td>
<td>chronic, treatment-resistant</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 g</td>
<td>27</td>
<td>8 weeks</td>
<td>prominent negative symptoms</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 g</td>
<td>12</td>
<td>12 weeks</td>
<td>treatment-resistant</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Glycine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potkin et al.</td>
<td>double-blind, placebo-controlled</td>
<td>30 mg/kg</td>
<td>20</td>
<td>6 weeks</td>
<td>prominent negative symptoms, PDS, treatment-resistant</td>
<td>NS</td>
</tr>
<tr>
<td>Evins et al.</td>
<td>double-blind, placebo-controlled</td>
<td>2 g</td>
<td>20</td>
<td>6 weeks</td>
<td>PDS, treatment-resistant</td>
<td>NS</td>
</tr>
<tr>
<td>Diaz et al.</td>
<td>double-blind, placebo-controlled</td>
<td>2 g</td>
<td>20</td>
<td>6 weeks</td>
<td>PDS, treatment-resistant</td>
<td>NS</td>
</tr>
</tbody>
</table>

↓ = exacerbation; NS = no significant change; − = negative; PDS = primary deficit syndrome
†Number of patients that completed the trial
Animal models

Animal models are useful tools that have the potential to further the understanding of the molecular, cellular, and environmental mechanisms involved in the pathogenesis of human disease and enable the development of novel therapies. However, creating adequate animal models of complex neuropsychiatric diseases, such as schizophrenia, is a particularly formidable challenge. Schizophrenia is a uniquely human disease of heterogeneous origin, characterized by many prominent symptoms that can not be measured in animals, including hallucinations, thought disorder, and delusions. Animals simply lack the ability to express many higher-order human functions in the realms of perception, thought, emotional experience, or language that are often affected in patients with schizophrenia. On the other hand there are certain behaviors that have been used as outcome measures in mice that resemble clinical features of schizophrenia, including social interaction, prepulse inhibition, and latent inhibition. Thus, in contrast to the full complex disorder, animals models can be used to represent certain endophenotypes associated with schizophrenia. Though often referred to as models of schizophrenia, they actually only model specific traits of the disease.

Considering the polygenic basis of schizophrenia, disruption of a single gene or neurotransmitter system is not likely to produce an animal model with complete construct validity. Instead, such approaches may be informative in determining the role of single genes and/or neurotransmitter pathways in normal brain function and their possible contribution to specific aspects of the disease. Recent advances in molecular technologies used to manipulate the mouse genome have provided vast opportunities for the development of models targeting genes implicated in schizophrenia pathophysiology. In principle, genetic strategies offer the advantage over pharmacological approaches of having greater selectivity
in its molecular targets and address the developmental components of disease. However, the application of genetic animal models is still at an early stage, and current models are often faced with compensatory changes that contribute to the adult phenotype. Furthermore, transgenic mice often involve disruptions of entire genes or large gene segments. Such genetic perturbations are rarely present in patients with schizophrenia, and instead single nucleotide polymorphisms within candidate genes have been associated with disease vulnerability.

Additionally, certain genes, such as G72, are unique to primates\textsuperscript{96}, while others are differentially expressed in humans compared to rodents\textsuperscript{222, 223}. Comparisons of genome wide binding sites for various transcription factors in humans and mice suggest that a substantial divergence of transcriptional regulation exists between mouse and human\textsuperscript{224, 225}. Many alternative splicing events that occur in humans have also been shown to be not conserved in the mouse\textsuperscript{226}. Therefore, although animal models can serve to investigate pathophysiological mechanisms and can predict antipsychotic efficacy, there are limitations to the extent these models can replicate the human condition.

**Animal models based on diminished NMDAR function**

Consistent with the psychotomimetic effects of NMDAR antagonists in humans, non-competitive inhibition of the NMDAR in animals produces a range of behavioral impairments that are reminiscent of the symptoms of schizophrenia\textsuperscript{227}. Behavioral abnormalities following acute or chronic treatment with an NMDAR antagonist in rodents include locomotor hyperactivity, information-processing disturbances, social approach impairments, and deficits in reversal learning and working memory\textsuperscript{227-233}. Similarly,
administration of PCP in non-human primates produces impairments in prepulse inhibition\textsuperscript{234}, social behaviors\textsuperscript{235}, and working memory\textsuperscript{236}. Chronic PCP treatment followed by a withdrawal period has also been shown to induce enduring cognitive deficits, along with reduced dopaminergic utilization in the prefrontal cortex\textsuperscript{17}. Furthermore, repeated exposure to PCP has been shown to induce neurodegeneration throughout the brain\textsuperscript{237}, which may resemble some of the neuroanatomical changes associated with schizophrenia\textsuperscript{21, 238}.

To address neurodevelopmental aspects of schizophrenia, models that involve perinatal treatment with NMDAR antagonists have been developed\textsuperscript{227}. These models test the hypothesis that viral or environmental insults occurring during the late second trimester of pregnancy, a period important to the development of the fetal CNS, subsequently increases the likelihood of developing schizophrenia in adulthood\textsuperscript{238}. In rats, the corresponding period is in the first two weeks of postnatal life\textsuperscript{239}, and administration of NMDAR antagonists during this time results in an increase in transient neuronal apoptosis\textsuperscript{240}. Early exposure to NMDAR inhibitors leads to the emergence of several schizophrenia-related behaviors in adult animals, including deficits in information-processing\textsuperscript{241}, enhanced sensitivity to NMDAR blockers and dopamine-releasing stimulants\textsuperscript{31, 242}, and impairments in working and reference memory\textsuperscript{243}. Additionally, perinatal administration of NMDAR antagonists induces enduring alterations in hippocampal NR1 subunit expression\textsuperscript{244}, mesolimbic dopamine receptor binding\textsuperscript{245}, synaptogenesis in the hippocampus\textsuperscript{246}, and decreases the number of cortical parvalbumin-positive GABAergic neurons in adult rats\textsuperscript{31}. Recently, oligodendrocyte differentiation and myelination have also been found to be affected by PCP treatment in developing rats\textsuperscript{247}, consistent with the white matter abnormalities that have been observed in
schizophrenia. Thus, early changes in NMDAR function produce lasting changes relevant to schizophrenia pathophysiology.

Since schizophrenia is a heritable disease proposed to involve abnormalities in the glutamatergic system, genetic animal models of aberrant NMDAR function have been established. These models also have the advantage of reproducing the chronic and developmental nature of NMDAR hypofunction theorized to occur in schizophrenia. Mice with complete and global loss of the NMDA-NR1 subunit die neonatally. However, targeted ablation of the NR1 subunit in the dentate gyrus of the hippocampus results in spatial working memory impairments, while loss of NR1 in hippocampal CA3 pyramidal cells results in deficient associative memory recall in adult mice. Mice with a 95% reduction in normal levels of NR1 show abnormalities in CNS development, disrupted sensorimotor gating, impaired social and sexual interactions, and increased spontaneous locomotor activity, stereotypy, and sensitivity to amphetamine. Alternatively, overexpression of the NMDA receptor composed of NR1-NR2B subunits in the mouse forebrain enhances NMDAR-dependent synaptic potentiation and produces improvements in learning and memory.

Although these studies support that disturbances in the NMDAR produce phenotypes that are potentially relevant to the symptoms of schizophrenia, they do not directly examine the effects of modulating the NMDAR glycine site. Considering the growing evidence indicating abnormal D-serine availability and NMDAR glycine site function in the pathophysiology of schizophrenia (as described earlier), animal models investigating the effects of decreased occupancy and activation of the NMDAR glycine site may be more proximal to the neural changes proposed to occur in schizophrenia. Furthermore,
examination of such animal models may uncover novel targets for the treatment of this disease.

**Hypothesis**

It is theorized that the NMDAR glycine site and D-serine modulatory enzymes have an important role in the pathophysiology of schizophrenia. Alterations that lead to a decrease in D-serine availability or occupancy of the glycine site are hypothesized to contribute to NMDAR hypofunction and could potentiate symptoms related to schizophrenia. In contrast, diminished D-serine catabolism and enhancements in glycine site agonists are predicted to be therapeutic.

Using pharmacological and genetic animal models, several approaches have been taken to investigate this hypothesis:

1) The beneficial effects of D-serine and of the indirect glycine site modulator ALX-5407 will be examined in mouse models relevant to schizophrenia and compared to the classical atypical antipsychotic, clozapine. It is proposed that D-serine and ALX-5407 will produce similar favorable effects as clozapine, and will reverse deficits related to MK-801-induced NMDAR hypofunction.

2) *Grin1*<sup>D481N</sup> mutant mice, which have a five-fold reduction glycine site affinity<sup>256</sup>, will be behaviorally characterized. These mice are predicted to demonstrate behavioral phenotypes that are relevant to schizophrenia, particularly to the
negative and cognitive symptoms. Abnormal behaviors in these animals are predicted to be reversible by D-serine.

3) A mutation in the Srr gene is predicted to lead reduced levels of D-serine. Biochemical, behavioral, and pharmacological responses will be examined. These mice are expected to display schizophrenia-like behaviors that are normalized by D-serine or clozapine.

4) Mutant mice that lack DAO activity and display enhanced D-serine levels \(^{257-259}\) will evaluated in spatial learning and extinction tasks that are relevant to schizophrenia and anxiety syndromes. It is predicted that these mice will display improvements in NMDAR-dependent tests. Enhanced performance will be confirmed by pharmacological administration of exogenous D-serine.

The overall aim of these approaches is to investigate the glutamatergic theory of schizophrenia and progress knowledge on the role of NMDAR glycine site, Srr, and DAO in the pathophysiology of this disorder. The study of genetic animal models will permit greater understanding on the effects of altered Srr and DAO expression, and may also identify novel therapeutic targets for the treatment of schizophrenia and other psychiatric disorders. Research involving exogenous administration of D-serine and GlyT-1 inhibitors will further determine the efficacy of these NMDAR-enhancing compounds and aid in determining their utility as putative antipsychotics.
CHAPTER 2

Experimental Procedures
Animal subjects

All animal procedures were approved by the Animal Management Committee of Mount Sinai Hospital and complied with the requirements of the Province of Ontario Animals for Research Act 1971 and the Canadian Council on Animal Care. Groups of 3-5 littermates were housed by sex in separately ventilated, filtered polycarbonate cages. Animals were given ad libitum sterile food (Purina mouse chow) and water, except where stated. The vivarium was maintained under controlled temperature (21ºC ± 1ºC) and humidity (50-60%), with a 12-h diurnal cycle (lights on: 0700-1900). All studies were conducted on experimentally naïve mice.

C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were acclimatized to the animal colony at least 1 week prior to testing. C57BL/6J mice were 8-16 weeks of age.

Grin1D481N mice were generated by site-directed mutagenesis and homologous recombination, and derived from founders generously provided by Dr. M. Pauly-Evers, Hoffman-La Roche Ltd. (Basel, Switzerland). The Grin1D481N mutation (aspartate to asparagine substitution at amino acid 481) is in the NR1 glycine-binding region and confers an approximately five-fold lower co-agonist affinity in the homozygous state. The D481N mutation was confirmed in our Grin1 stock by sequencing a 450 bp PCR product (Primers: 5’-ATGTCAAGCCCCACAATGAGT-3’ and 5’-CCTGCTCCACGTCTACCTAA-3’) using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Grin1D481N mice were backcrossed 11 generations onto the C57BL/6J strain and then bred from heterozygous intercrosses to attain experimental subjects. Grin1D481N mice were used in the experiments in chapter 4 (12-16 weeks of age) and chapter 6 (16-20 weeks of age).
Genotyping was completed using a PCR-amplicon restriction endonuclease protocol that detected the presence of an MsiI (Fermentas, Burlington, ON, Canada) restriction site in animals carrying the Grin1<sup>D481N</sup> mutation.

To identify a mutation in the Srr gene, the temperature gradient capillary electrophoresis (TGCE) heteroduplex detection method was used to screen genomic fragments spanning exons of the Srr gene for N-nitroso-N-ethylurea (ENU)-induced mutations in 7502 mice, as described<sup>261</sup>. In exon 9, a mutation was identified using the primer pair: 5’-GGCATTGTAAACAGAACCCTG-3’ and 5’-CTGCAACCAACCAAAAACTC3-3’. Direct sequencing with an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA) and data analysis with the Sequencher program (Gene Codes, Ann Arbor, MI, USA) confirmed the presence of a nonsense mutation at amino acid 269. Animals carrying the Srr<sup>Y269*</sup> mutation were bred with C57BL/6Jcl mice obtained from CLEA Japan (generation 2; Tokyo, Japan), and C57BL/6J mice purchased from The Jackson Laboratory (generation 3-8; Bar Harbor, ME, USA). After the Srr<sup>Y269*</sup> mice were backcrossed for 8 generations, experimental subjects were derived from heterozygous intercrosses. Mice were tested at 9-12 weeks of age, except where stated. Genotyping Srr<sup>Y269*</sup> mice involved the amplification of a 363-bp PCR product using the primer pair: 5’-GCTACCAGTCTAAACTGAAAGGAGA-3’ and 5’-CCAGCAGTCGGCTCAATG-3’. PCR products from homozygous mutant mice lacked an HpyCH4V (Fermentas, Burlington, ON, Canada) restriction site.

Dao1<sup>G181R</sup> mice were obtained from the Konno laboratory that initially identified a spontaneous missense mutation (glycine to arginine at amino acid 181) in the Dao gene of the ddY strain resulting in a complete loss of DAO activity<sup>257,258</sup>. The Dao1<sup>G181R</sup> mutation
was transferred onto a C57BL/6J genetic background using a marker-assisted speed congenic strategy\(^2\). The resultant \(\text{Dao1}^{G181R}\) mice contained >99% of the C57BL/6J genome after 6 generations of backcrossing. Experimental subjects were then bred from heterozygous intercrosses of \(\text{Dao1}^{G181R}\) mice and tested at 11-16 weeks of age. Animals carrying the \(\text{Dao1}^{G181R}\) mutation were genotyped using a PCR-amplicon restriction endonuclease protocol that involved the amplification of a 263-bp PCR product (5’-TGATGTACGAAGCTGGAGGACA-3’ and 5’-TGTAGTGGCACCAGCTTT-3’), which lacked an \(HpaI\) (Fermentas, Burlington, ON, Canada) restriction site in the homozygous state.

**Behavioral studies**

Behavioral testing was performed during the light phase between 0900 and 1700 h. Experiments were randomized with regard to day and drug treatment, and sex-balanced, except where stated. Since no sex differences were found in the measured behaviors, male and female data were pooled for greater subject numbers. Experimenters were blind to genotype. Prior to experiments, mice were left undisturbed in the room for at least 30 min to allow for acclimatization. Where appropriate, the experimental equipment was cleaned with 70% ethanol between each subject.

**Prepulse inhibition (PPI)**

Acoustic startle response and PPI were tested using 4 sound-attenuating chambers (ENV-022s; MED Associates, St. Albans, VT, USA). Each chamber contained an acoustic stimulator (ANL-925), and a load cell platform with a transducer amplifier (PHM-255A and
PHM-250B) to detect animal movements. Chambers were also equipped with a fan and a red light. Mice were placed into ventilated holders (ENV-263) that were attached to the platform of each chamber. Data acquisition and analysis employed the MED Associates software (Startle Reflex package).

Testing sessions began by individually placing mice into the startle chambers. Mice were given a 15-min acclimatization period to background noise (65 dB). Afterwards, subjects were presented with a series of five startle-pulse-alone (P) trials, each comprised of a single white noise burst (120 dB, 40 ms). This was followed by a series of trials consisting of no stimulus (background noise, 65 dB), a startle-pulse-alone, or one of three prepulse intensities (69, 73, and 81 dB, 20 ms) presented 100 ms before a startle-pulse (120 dB, 40 ms). This series of trials was given in 10 blocks each containing all five trial types (no stimulus, P, 69 dB + P, 73 dB + P, 81 dB + P) in pseudorandom order. Finally, an additional series of five startle-pulse-alone trials were presented. Intertrial intervals were between 12 to 30 s. The peak startle activity for each trial was recorded. PPI was expressed as the reduction in startle amplitude in prepulse + pulse trials compared to startle-pulse-alone trials. The % PPI for each prepulse intensity was calculated according to the following formula: %PPI = 100-(startle response on prepulse trials / startle response on startle-pulse-alone trials) × 100.

To measure startle reactivity, mice were acclimatized to the testing chamber for 15 min prior to the presentation of startle stimuli of varying intensities (70-120 dB), with a 25-ms duration and an inter-stimulus interval of 25-30 s. Startle stimuli were presented in three blocks each composed of two demonstrations of the 11 stimulus intensities given in pseudorandom order. The average startle amplitude for each stimulus intensity was calculated from the three blocks.
**Latent Inhibition (LI)**

The LI procedure was adapted from a previously described protocol\textsuperscript{263, 264}. LI experiments were conducted in 3 sound-attenuating chambers (ENV-022M, MED Associates, St. Albans, VT, USA) that were each illuminated with a light (ENV-221CL), ventilated with a fan (background noise 68 dB), and equipped with a speaker (ENV-324M). The conditioning chambers (ENV-307W), within the sound-attenuating chambers, were made of clear Plexiglas (21.6×17.8×12.7 cm) and had removable floors that consisted of a smooth metal plate or a metal grid floor. A foot shock (0.37 mA intensity, 1 s duration) could be delivered through the grid floor by a shock generator (ENV-414). Licks of water from a sipper tube were detected by a lickometer (ENV-350CM) connected to a computer. The MED Associates software (MED-PC, St. Albans, VT, USA) that controlled the experimental parameters and data acquisition was connected to the chambers via an interface package (DIG-716P1 and ANL-926).

Mice were weighed and water was removed from home cages 24 hours prior to the start of the LI procedure. Throughout the experiment, weights were monitored to ensure animals did not lose more than 20% of their original body weight. During the 5-day pretraining phase mice were trained to drink from the sipper tube in the LI chamber. Animals were given 5 min to acclimatize to the chamber without access to water, followed by a 15-min period with free access. The latency to the first lick and the number of licks were recorded.

The LI procedure was conducted on days 6-9 and was composed of preexposure, conditioning, baseline drinking, and testing stages. Preexposure (day 6) and conditioning
(day 7) involved placing all mice in the testing chamber without access to water and began with a 5-min acclimatization period. During the preexposure phase, half the animals received 40 white-noise stimuli (85 dB, 10 s duration) interspaced 60 s apart (preexposed), and the other half received no tone stimulus (nonpreexposed). In the conditioning stage, all animals received 2 or 4 noise-shock pairings interspaced by 5 min, where presentation of a 10-s white noise stimulus was immediately followed by a foot shock (0.37 mA intensity, 1 s duration). After both the preexposure and conditioning phases water was administered in the home cages for 15 min. Day 8 was the baseline drinking stage, where animals received free access to the water for 15 min, as in the pretraining phase. The baseline drinking day was necessary to eliminate any context-shock association, and to ensure the animals would continue drinking from the sipper tube. Animals that completed less than 100 licks on this day were excluded from the experiment. The testing stage (day 9) involved a 5-min acclimatization period followed by access to the water. The chamber was silent from lick 0 to 75, but during lick 76 to 101 a continuous white noise stimulus (85 dB) was presented. The latency for the first lick, the time between lick 50-75 (before noise – A period), and the time between lick 76-101 (during noise – B period) was recorded. Suppression of the lick response was expressed as the suppression ratio A/(A+B). Latent inhibition is present when preexposed animals have a higher suppression ratio (lower tone response) than nonpreexposed animals on testing day.

**Social affiliations**

The social affiliations task was adapted from previously described studies\(^{265, 266}\). The social affiliations task was conducted in a clear Plexiglas box (61.5 cm length \(\times\) 46 cm
width×23 cm height) divided into three interconnected chambers (outer chambers: 19 cm length, central chamber: 23.5 cm length). The outer chambers were identical to each other and divided from the central chamber by clear Plexiglas partitions (17.5 cm width×23 cm height) that each had a centrally placed opening (11 cm width×23 cm height) and a retractable chamber divider. Transparent Plexiglas cages with a cylindrical shape (13 cm height, 8 cm diameter) were used to contain the stranger mice and were perforated with evenly distributed holes (1 cm diameter). Throughout the experimental sessions, the cages were located at the center of each outer chamber and permitted auditory, visual, and olfactory investigation.

Only male animals were used in the social affiliations experiments. The stranger mice used were age-matched, male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) that had never been in contact with the test subjects. At the beginning of each experimental session, the test mouse was placed in the central chamber and was allowed to freely explore for 10 min. Data were not recorded during this habituation period. Afterwards, the experimenter placed a stranger mouse (stranger 1) in one of the cages and handled the other empty cage similarly. The cage and outer chamber containing the stranger mouse was alternated across subjects. After the placement of the stranger mouse, the test subject explored the social apparatus for 10 min (sociability phase). The test mouse was considered to be in a chamber if its head and two front paws had entered the chamber. The amount of time spent investigating each chamber, the number of entries into each chamber, freezing, and grooming were scored using the Observer 5.0 software (Noldus, Wageningen, The Netherlands) and video recorded. Sociability was assessed by comparing the amount of time
spent in the chamber with the caged stranger mouse to the amount of time spent in the opposite empty-cage chamber.

Preference for social novelty was also examined. Immediately after the sociability assessment detailed above, a second unfamiliar mouse (stranger 2) was placed beneath the previously empty cage (social novelty phase). The test mouse could now explore the central chamber, the chamber containing the initial stranger (stranger 1; now familiar), or the chamber containing a novel stranger (stranger 2) for a period of 5 min (chapter 4) or 10 min (chapter 5). All other parameters and measures were as described above in the sociability phase.

In chapter 4, an additional experiment was performed in which there were three sessions. Session 1 and 3 were the previously described sociability and social novelty phase. Session 2 was conducted exactly as session 1, except a new unfamiliar mouse (stranger 2) was caged in the chamber that had previously been empty in session 1.

**Olfactory test**

Olfactory acuity was assessed as previously described\(^{267}\). Male mice were given a 24-h food deprivation period before testing. In the experiment, clean polycarbonate cages (30×17×12 cm) with fresh corncob bedding were used for each subject. One piece (approximately 1×1×1 cm) of Lab Diet rodent chow (PMI Nutrition International, Brentwood, MO, USA) was buried in a random location beneath an evenly distributed layer of corncob bedding (2.5 cm in depth). The latency to find the buried food was recorded, with a maximum period of 10 min.
**Spatial and nonspatial object recognition**

The spatial and nonspatial discrimination task was performed in a transparent Plexiglas open field (41×41×31 cm) equipped with infrared beams to detect locomotor movements (model 7420/7430; Ugo Basile, Comerio, Italy). The five objects used in this task differed in shape, color, and material (approximately 7×5×5 cm). Animal behavior was recorded and analyzed using The Observer 5.0 (Noldus, Wageningen, The Netherlands).

The testing procedure was adapted from previously described protocols\(^{268-270}\). Only male mice were used, as sex differences have been reported for spatial novelty responses in C57BL/6J mice\(^{268}\). On test day, each mouse was individually placed in the center of the empty arena for a 5-min session, and locomotor activity (beam breaks) was recorded. The mouse was then placed in a holding cage for 2 min. Four objects were placed in specific positions near each corner of the arena (5 cm from corner walls). The mouse was returned to the center of the arena and allowed to explore the objects for three (chapter 5) or four (chapter 4) continuous 5-min sessions (habituation phase). Habituation to object exploration was measured by recording the time spent exploring the objects across the sessions. A mouse was considered to be exploring an object if its snout was in contact with the object. Duration of locomotor activity was also measured during the habituation sessions and in subsequent sessions. At the end of the habituation phase, the mouse was again placed in the holding cage for 2 min and the position of two objects (NW and SE or NE and SW) was switched to assess response to a spatial change. During the switch, all four objects were touched by the experimenter and objects that were moved were counterbalanced within groups. The mouse was returned to the arena, and the time spent exploring the displaced and nondisplaced objects was recorded for 5 min (spatial change phase). Reaction to a spatial change was
assessed by comparing the mean time spent exploring the displaced (DO) and nondisplaced object (NDO) categories.

Reactivity to a nonspatial change was also examined. Directly after the spatial change phase, the test subject was returned to the holding cage for 2 min, during which one of the familiar nondisplaced objects in the arena was replaced by a novel object in the same location. The mouse was returned to the center of the arena for a 5-min period (nonspatial change phase). Measurements were taken as described for the previous phase, and the response to nonspatial change was evaluated by considering the mean time spent exploring the novel object (NO) and the three familiar objects (FOs).

**Neurological assessment**

A neurological examination was performed as described\(^{267, 271}\), and involved an evaluation of sensory functions and reflexes to detect any gross abnormalities. Weights were measured in mice that were 8, 12, and 18 weeks of age. All other measures in this assessment were completed on mice that were 8 weeks of age. The presence and condition of fur and whiskers was assessed visually. The eye-blink reflex was examined by approaching an eye with the end of a cotton swab, causing the immediate blink of that eye. The ear-twitch reflex was examined by approaching an ear from behind with a cotton swab that would then touch the pinna, resulting in immediate movement of that ear. The whisker-twitch reflex was examined by gently touching the ends of one set of vibrissae, resulting in cessation of vibrissae movement and a head turn towards the touched side. The righting reflex was examined by turning the mouse on its back, causing the immediate regain of an upright posture. Vision was assessed using the visual placing test\(^{267}\) (chapter 4 and 5). Vision was
considered to be normal in mice (>80% group criteria) that would reach for a passing table surface, after being lowered 15 cm above and 4 cm out from the table surface.

**Accelerating rotarod**

Performance in an accelerating rotarod task and motor learning was examined using a modification of a previously described protocol. The rotarod apparatus (Economex Rotarod; Columbus Instruments, Columbus, OH, USA) had a ribbed rotating axle (3 cm diameter) that was situated 30 cm above a plastic surface. A maximum of four mice could be tested simultaneously with each mouse being separated by an opaque wall (30 cm width×60 cm height). Mice were placed on the rod facing away from the experimenter and initially allowed to acclimatize to the stationary rod for 60 s. The axle was then set at a constant speed of 5 rpm for 90 s. Afterwards, the axle speed (starting at 5 rpm) was increased by 0.1 rpm/s and the latency to fall off the axle was recorded for each mouse, with a maximum of 360 s in the acceleration mode. On each day, 3 trials were completed with a 1-h intertrial interval. Motor coordination and balance was evaluated by comparing the mean latencies to fall from the rod (average of the 3 daily trials). Motor learning was determined by observing an improved latency on the last compared to the first day (motor learning ratio = latency day 3 / [latency day 1 + 3]). Impaired performance in the stationary and constant speed sessions was considered to be an early indicator of motor abnormality.

**Locomotor activity**

Locomotor activity was measured for 30 min (5-min bins) in a transparent Plexiglas open field (41×41×31 cm) equipped with infrared beams to detect horizontal movements
(model 7420/7430; Ugo Basile, Comerio, Italy). The testing period began by placing a mouse individually at the center of the automated activity cage. The mouse was allowed to freely explore the apparatus for the duration of the test.

**Forced swim test**

Floating duration in the forced swim test was evaluated, as described. Mice were place into a cylinder (25 cm height, 18 cm diameter) containing water (26°C) at a depth of 18 cm for a duration of 6 min. The time spent immobile, as defined by floating in an upright position with only minimal movements necessary for the head to remain above water, was recorded during the last 4 min of the test period. The water was changed between each subject. Testing sessions were video recorded, and data was collected and analyzed using an event-recording program (Observer 5.0; Noldus, Wageningen, The Netherlands).

**Elevated plus-maze**

Anxiety-like responses were measured in the elevated plus-maze, as described. The apparatus consisted of two open arms (25×5 cm; 70 lx) and two closed arms (25×5×30 cm; 1.3 lx) extending from a central platform (5×5 cm) and elevated 50 cm from the ground. The floor of the arms was made of white Plexiglas and the walls of the closed arms were made of black Plexiglas. Similar arms were opposite to each other and at a 90° angle from dissimilar arms. The test mouse was placed in the central area facing an open arm and allowed to explore the apparatus for 5 min. The number of entries and the time spent in the open arms, closed arms, and central platform was recorded and analyzed using the Observer 5.0 program (Noldus, Wageningen, The Netherlands) and videotaped. An entry was defined
as placing all four paws within one arm of the maze. Anxiolytic effects were assessed by an increase in \( \% \) open arms time \((\text{open arm time/ total time on apparatus} \times 100)\) and in \( \% \) open arm entries \((\text{open arm entries / total arm entries} \times 100)\), whereas anxiogenic effects were indicated by a decrease in these measures. Total number of entries (open + closed arm entries) was used as a measure of overall motor activity.

**Morris water maze**

The Morris water maze (MWM) consisted of a white Plexiglas, cylindrical pool (1.2 m diameter) that was filled with opaque water \((26^\circ \text{C} \pm 1^\circ \text{C})\). The pool was arbitrarily divided into four equal quadrants: northeast, northwest, southeast, and southwest. The circular escape platform (10 cm diameter) was made of clear Plexiglas. Distal visual cues were fixed on each wall \( \sim 1 \) m from the pool edge. Activity in the water maze was recorded using a CCD camera on the ceiling above the center of the pool attached to an automated tracking system (HVS Image, Twickenham, Middlesex, UK) that extracted and stored the X-Y coordinates of the subject every 0.01 s. The HVS Water 2020 software (HVS Image, Twickenham, Middlesex, UK) was used to establish experimental parameters and analyze performance. Behavioral measures in the MWM included latency to target (s), path length (m), thigmotaxis (\% time wall hugging), swim speed (cm/s), floating (\% time), \% time within the target area, and number of platform crosses.

In some experiments (chapter 6), a larger MWM pool (1.85 m diameter) and escape platform (15 cm diameter) was used. This was done to increase the possible distance between platform locations in the delayed-matching-to-place experiments, and to increase the
difficulty of the task in the $\text{Dao}^{G181R}$ experiments. All other methodology described applies to both the small and large pool experiments.

Subjects were handled for 2 min/day on each of the 5 consecutive days prior to testing. Each MWM procedure began with a 1-day stationary visible platform task. Mice were given 4 trials with a ~1-h intertrial interval (ITI). In each trial, mice were released facing the pool wall from one of four pseudo-randomized locations (N, S, W, E) at the pool periphery. To escape, mice had to find a platform that was located at the center of a quadrant, 25 cm ($\text{Srr}^{Y269*}$ and $\text{Dao}^{G181R}$ experiments) or 30 cm ($\text{Grin}^{D481N}$ experiments) from the pool wall. In the visible trials, the platform was raised 0.5 cm above the water surface and demarcated with a 10 cm vertical pole. The maximum duration for a platform search was 90 s. Animals that found the platform remained on it for an additional 15 s, whereas unsuccessful animals were assigned a 90-s latency and gently placed onto the platform for 15 s. The acquisition phase began 1 day later, and lasted for four ($\text{Grin}^{D481N}$ experiments) or seven ($\text{Srr}^{Y269*}$ and $\text{Dao}^{G181R}$ experiments) consecutive days (4 trials/day, 1 h ITI). Each day was performed similarly to the visible platform task, except the platform was now submerged ~1 cm below the surface of the water (hidden). Retention of spatial memory was assessed in a 60-s probe trial occurred 24 h after the last acquisition trial (and also after acquisition day 5 in the $\text{Srr}^{Y269*}$ experiments). In the probe trials, the platform was removed and mice were released from the point furthest from the former platform location. Performance in the probe trial was quantified by examining the % time spent and number of crosses over the target platform area, centered over its former location.

Reversal learning and memory. The reversal experiments entailed 3 additional days of training (4 trials/day, 1 h ITI) that began 1 day after the acquisition probe. Reversal trials
were conducted as described in the above acquisition phase, with the exception that the platform was located in the center of the opposite quadrant. Memory for the new platform location was examined in a 60-s reversal probe that was assessed 24 h after the last reversal trial.

**Delayed-matching-to-place.** The delayed-matching-to-place (DMP) version of the MWM task consisted of a single day of visible platform training and 12 acquisition days, where the hidden platform was moved to one of 12 predetermined locations at the beginning of each day. The platform at each position was either 25 or 40 cm from the wall. Within a training day, the platform remained in the same position for four trials with an ITI of 45 min between all trials. All other parameters were conducted as described for the stationary hidden platform MWM task.

**Extinction.** In the MWM, extinction was assessed in the *Dao1*<sup>G181R</sup> mice and animals given D-serine treatments. The extinction trials were performed as described in the acquisition probe, and occurred on the following days after the last acquisition trial: 1, 3, 5, 7, 9, 11, 13, and 15 (48 h ITI). To verify that the *Dao1*<sup>G181R</sup> mutation or D-serine treatments did not impair memory duration, control groups of *Dao1*<sup>G181R</sup> mutant and D-serine-injected mice were tested for the first time 9 days after the completion of acquisition training. The D-serine-treated control group was injected in the home cage on the same days as those mice exposed to multiple probe trials.

**Fear conditioning**

The fear conditioning apparatus (MED Associates, St. Albans, VT, USA) consisted of 4 test chambers (25 cm height×30 cm width×25 cm length) that each had a grid floor and
were connected to a shock generator, amplifier, and speaker. Experimental parameters were controlled by automated fear conditioning software (FreezeFrame v. 1.6e, Actimetrics, Evanston, IL, USA) that also recorded freezing activity (presented as a % of total time). Prior to the fear conditioning procedure, subjects were handled for 2 min/day for 5 days.

Contextual and cued fear conditioning was conducted as previously described\textsuperscript{273, 274}. In the training phase, each chamber had a white curtain covering the front exterior and was cleaned with 70% ethanol that left an odor. All surfaces in contact with test subjects were carefully dried and odor cues were placed on a surface that was unattainable by the animals. The training phase began with a 2-min period in the chamber to monitor baseline activity. Afterwards, a 30-s auditory tone was delivered (3600 Hz, 95 dB), and in the last 2 s of tone presentation a continuous foot shock (0.75 mA scrambled) was administered. The animals were given an additional 30-s period (post-US) before being returned to their home cages. Freezing to the context was assessed for 5 min without tone or foot shock on the following days after training: 1 (24 h later), 3, 5, 7, and 13. The fear conditioning extinction sessions were conducted over days in order to resemble the extinction procedure completed in the MWM. The % time freezing specifically to the context was calculated for each subject \[\text{%time freezing to context} = \text{% time freezing during context exposure} - \text{%time freezing during training baseline period}\], and these values were averaged for each genotype\textsuperscript{275}. Freezing response to the tone cue was measured in an altered chamber 2 h after each contextual freezing session. Ventilation of the testing room, in addition to careful cleaning and drying of the chambers during this delay eliminated the odor used in the contextual freezing session. For the tone test, the sensory environment of the chamber was altered using a 1% acetic acid odor, a smooth white Plexiglas floor, a black curtain on the front exterior,
and a clear Plexiglas insert that gave the interior of the chamber a prism shape. These chamber alterations did not affect the tone frequency, but did reduce the tone amplitude by 2 dB. Animals were placed in the chamber and given 3 min to explore (pretone) followed by a 3-min exposure to the tone. The % time freezing specifically to tone was determined for each subject [% time freezing to tone = % time freezing during tone exposure – %time freezing during pretone period], and these values were then averaged for each genotype.

**Western blot analysis**

Dissected whole brain tissue was homogenized in 1.5 ml of RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Homogenates were centrifuged at 20,000 × g for 10 min at 4°C, supernatants were collected, and protein concentrations were measured by a Bradford assay (Bio-Rad, Hercules, CA, USA). Protein samples (50 μg) were suspended in loading buffer (Bio-Rad, Hercules, CA, USA) containing 2-mercaptoethanol (Sigma, Oakville, ON, Canada), incubated at 95°C for 5 min, and loaded onto an SDS-PAGE gel (Criterion; Bio-Rad, Hercules, CA, USA) along with 5 μl of MagicMark XP Western Protein Standard (Invitrogen, Burlington, ON, Canada) to be separated (100 V, ~2 h) using a Bio-Rad Criterion electrophoresis system (Bio-Rad, Hercules, CA, USA). Proteins were then electrotransferred (50 V, 2 h) onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 5% nonfat dry milk (Bio-Rad, Hercules, CA, USA) in TBS-T solution (TBS, 0.1% Tween-20) for ≥1 h, and then incubated with one of the following primary antibodies: mouse anti-Srr monoclonal antibody (1:500; BD Biosciences, Mississauga, ON, Canada), Srr polyclonal antibody, T-16 (1:100; Santa
Cruz Biotechnology, Santa Cruz, CA, USA), anti-PICK1 monoclonal antibody, clone L20/8 (1:1000; UC Davis/NINDS/NIMH NeuroMab Facility, Davis, CA, USA), NR1 (1:500; Millipore, Billerica, MA, USA), GluR1 (1:100; Calbiochem, Gibbstown, NJ, USA), GluR2 (1:100; Calbiochem, Gibbstown, NJ, USA), glycine transporter-1 (1:500; Millipore, Billerica, MA, USA), and D-amino acid oxidase (1:1000; Nordic Immunological, Tilburg, The Netherlands). Srr antibodies were specific to epitopes in the N-terminus region.

Incubation with the primary antibody was completed in blocking solution overnight at 4°C (Srr, PICK1) or for 2 h at room temperature (NR1, GluR1, GluR2, GlyT-1, DAO). Following washes with TBS-T (3 × 10 min), the membrane was incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse IgG (1:5,000 in blocking solution; GE Healthcare, Piscataway, NJ, USA), anti-rabbit IgG (1:5,000 in blocking solution; GE Healthcare, Piscataway, NJ, USA), anti-goat IgG (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was washed in TBS-T (3 × 10 min) and then processed for chemiluminescence using a Western blotting detection kit (GE Healthcare, Piscataway, NJ, USA). Equal loading was confirmed by immersing the membrane in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) prior to incubation with a rabbit anti-β-tubulin III polyclonal antibody (1:20,000 in blocking solution for 1 h; Sigma, Oakville, ON, Canada). Exposure of the membrane to photographic film permitted for visualization of protein bands that were then quantified by densitometric analysis using the Image J 1.41 software (http://rsbweb.nih.gov/ij/). Each densitometric value was normalized to its respective β-tubulin III loading control.

Real time RT-PCR
The mRNA levels of Srr, and PICK1 were quantified in Srr\textsuperscript{Y269*} mice using real-time RT-PCR, as described\textsuperscript{277}. Total RNA was isolated from whole brain using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s instructions. RNA yield was quantified by UV spectrophotometry (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was verified using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

For real-time RT-PCR reactions, total RNA samples (2 μg) were initially DNase treated (Turbo DNase, Ambion, Austin, TX, USA) for 30 min at 37°C, and then incubated with 5 mM EDTA at 75°C for 10 min to inactivate DNase I. The DNase-treated RNA was used as a template to generate a cDNA archive using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Following cDNA generation, gene expression was assayed in triplicate on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Genes were amplified with Platinum Taq (Invitrogen, Burlington, ON, Canada) and quantitative RT-PCR reagents (Invitrogen, Burlington, ON, Canada), and expression was normalized to housekeeping genes (18s rRNA, γ-actin, histone 3a). Three housekeeping genes were utilized to rule out potential genotype specific effects in housekeeping gene expression. Primer sequences for quantitative analysis of the following genes were: Srr (5’-AGGCCCTGAAACCTAGTGTGAA-3’, 5’-TTCATTCTCCCCCACACCA-3’), PICK1 (5’-CGAGGAATACAGCTGCATTGC-3’, 5’-CGCAGAATGAGGCGGTACTC-3’), 18s rRNA (5’-GTAACCCGTTGAACCCCATT-3’, 5’-CCATCCAATCGGTAGTAGCG-3’), γ-actin (5’-CTTCCCCACGCCATCTTG-3’, 5’-CCCGTTCAGTCAGGATCTTCAT-3’), and histone 3a (5’-CGCTTCCAGAGTGCAGCTATT-3’, 5’-ATCTTTCAAAAAAGGCCAACCGAT-3’).
Srr activity

The procedure to measure activity of Srr in whole brain was adapted from a previously described protocol. Brain samples were homogenized in 5 volumes of ice-cold buffer A [50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 15 μM pyridoxal L-phosphate (PLP), 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin] and centrifuged at 20,000 × g for 30 min at 4°C. Supernatants were measured for protein concentrations and equalized prior to a two-step ammonium sulfate purification. Saturated (NH₄)₂SO₄ was added to a final concentration of 20%; samples were mixed by gentle rotation for 2 h at 4°C, then centrifuged at 14,000 × g for 10 min at 4°C to remove the pellets. Afterwards, the concentration of (NH₄)₂SO₄ in the supernatants was raised to 45%, and samples were rotated for 1 h at 4°C, then left unstirred at 4°C overnight. Pellets were collected by centrifugation at 14,000 × g for 10 min at 4°C, then resuspended in 320 μl of buffer B (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1 mM DTT, 15 μM PLP). Samples were dialyzed against 1000 ml of buffer B for 2 h at 4°C, and protein concentrations were measured in each sample.

Srr reactions were performed with enriched L-serine as a substrate. To minimize the levels of the D-serine that contaminates virtually all L-serine preparations, commercially obtained L-serine (Sigma, St. Louis, MO, USA) was reacted with porcine DAO (Calzyme Laboratories, San Luis Obispo, CA, USA) as follows: Up to 20 μmol of L-serine was combined with 1.5 U/ml porcine DAO, 3 U/μl catalase (Sigma, St. Louis, MO, USA) and 84 μg/ml flavin adenine dinucleotide (Calbiochem, Gibbstown, NJ, USA) in buffer C (0.15 M Tris-HCl, pH 8.3); the mixture was incubated at 37°C overnight, then at 95°C for 10 min. The
supernatant was collected after centrifugation at 17,000 × g for 10 min. Removal of D-serine was confirmed by a D-serine chemiluminescent assay (below). This enriched L-serine was combined with 1 mM ATP, 1 mM MgCl₂, and 1 mM CaCl₂ in buffer B. Srr reactions were initiated by addition of brain protein samples (100 μg), incubated at 37°C for 2 h, and then stopped by incubation for 5 min at 100°C.

A chemiluminescent assay was then used to measure D-serine levels in the Srr reactions. Buffer D (100 mM Tris-HCl, pH 8.8, 50 mM NaCl) containing 0.1 U horseradish peroxidase (Sigma, St. Louis, MO, USA), 0.8 nmol luminol (Sigma, St. Louis, MO, USA), and 0.048 nmol flavin adenine dinucleotide (Calbiochem, Gibbstown, NJ, USA) was diluted into each Srr reaction at a 1:5 ratio. A Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) was used to detect the production of H₂O₂ before and after the addition of 0.002 U R. gracilis DAO (courtesy of L. Pollegioni, U. Insubria, Varese, Italy). Triplicates of each sample were measured, and the content of D-serine was quantified using a calibration curve of D-serine standards.

Chemiluminescent assay for Dao₁<sup>G181R</sup> experiments

To detect D-serine levels in the hippocampus and whole cortex of Dao₁<sup>G181R</sup> mice, a chemiluminescent assay was conducted. Brain samples were homogenized in ice-cold buffer (50 mM Tris-HCl, pH 8.8, 10 mM KCl), and then centrifuged at 14,000 × g for 10 min at 4°C. From the supernatant, protein concentrations were measured and standardized. Samples were heated at 100°C for 20 min to eliminate endogenous DAO activity and then cooled to 4°C. The samples were then processed as described above in the chemiluminescent assay for the Srr reactions.
High-performance liquid chromatography (HPLC)

To measure D-serine concentrations in whole brain and brain regions, a HPLC procedure was adapted from a previously described protocol\textsuperscript{278}. Brain samples were homogenized in 5 volumes of ice-cold double distilled water. An aliquot was mixed with 100\% methanol to give a final dilution of 60× and then centrifuged at 12000 × g for 4 min at 4°C. A 5 μl aliquot of the supernatant was mixed with 5 μl of the derivatizing reagent (2 mg N-isobutyryl-L-cysteine and 1 mg o-phthalaldehyde dissolved in 0.1 ml methanol, followed by addition of 0.9 ml 0.1 M sodium borate buffer), and then was placed into a sample management system (Waters Alliance 2690XE, Waters Corp., Milford, MA, USA). HPLC separation was achieved on a Symmetry C18 column (4.6 mm×150 mm; 3.5 μm particle diameter) coupled with a guard column of the same stationary phase (Waters Corp., Milford, MA, USA). The column heater was set at 30°C and the sample cooler was held at 4°C. To separate the derivatized amino acids of interest, a gradient was established from equal parts of solvent A (850 ml of 0.04 M sodium phosphate buffer and 150 ml methanol, pH 6.2) and B (670 ml of 0.04 M sodium phosphate buffer, 555 ml methanol and 30 ml tetrahydrofuran, pH 6.2) to only solvent B by ~45 min, with a flow rate of 0.5 ml/min. The run time was 60 min for column washout and equilibrium, and 30 min to elute all compounds. A Waters 2475 fluorescence detector (Waters Corp., Milford, MA, USA) was used to quantify the eluted compounds (excitation 344 nm; emission 433 nm).

Drugs
D-serine, L-serine, ALX-5407 ((R)-N-[3-(4’-fluorophenyl)-3(4’-phenylphenoxy)propyl]sarcosine hydrochloride), (+)-MK-801 hydrogen maleate, Ro 25-6981 were purchased from Sigma (Oakville, ON, Canada). Clozapine and L-701,324 (7-chloro-4-hydroxy-3(3-phenyoxy)phenylquinoline-2-(H)-one) were purchased from Tocris (Ellisville, MO, USA). D-serine, L-serine, and MK-801 were dissolved in a saline (0.9% NaCl) solution. ALX-5407 was dissolved in 75% ddH2O / 25% 2-hydroxypropyl-β-cyclodextrin, pH adjusted to ~6 using 1N NaOH. Clozapine and Ro 25-6981 were dissolved in 0.9% NaCl with 0.3% Tween. L-701,324 was dissolved in a solvent containing 25% polyethyleneglycol-300, and slightly alkalinized with 1N NaOH. D-serine (300, 600, 900 mg/kg), L-serine (600 mg/kg), and Ro 25-6981 (5 mg/kg) were injected subcutaneously. ALX-5407 (1, 10, 15 mg/kg), clozapine (0.75, 3.0, 6.0 mg/kg), and L-701,324 (5mg/kg) were injected intraperitoneally. MK-801 (0.05, 0.1, 0.15, 0.2, 0.3, 0.6, 1.0 mg/kg) was injected intraperitoneally 15 min before the experiments in chapter 3, or injected subcutaneously 30 min prior to testing in chapter 5. D-serine was administered 20 min before testing in all experiments, except for in the SrrY269* and Dao1G181R studies where D-serine was given 30 min before testing. Additional injection-testing intervals were 20 min for L-serine, 120 min for ALX-5407, 45 min for L-701,324, and 30 min for clozapine and Ro 25-6981. All drugs were administered at volume of 10ml/kg. Drug doses were chosen based on work completed during this thesis279-281, pilot experiments, and other behavioral studies43, 58, 192, 228, 229, 232, 253, 282-286.

In LI experiments all drugs were administered in the preexposure and conditioning stages. In the MWM experiments with mice from the Grin1D481N line (Figure 6.1 and 6.2), D-serine was given before the first trial of each training day, and no drugs were given before the probe trials. For the extinction experiment involving drug treatments (Figure 6.6), D-serine
was administered before each probe trial, and no treatments were given during visible platform or acquisition training sessions.

**Statistical analysis**

Statistical analyses were performed using Statistica (Statsoft, Tulsa, OK, USA). Biochemical and behavioral data were analyzed using one-way, two-way, or repeated-measures (RM) ANOVA with the appropriate between-subjects and within-subjects factors. Significant main effects or interactions were followed by Fisher’s least significant difference (LSD) post hoc comparisons. Significance was set at $p < 0.05$. 
CHAPTER 3

Modulators of the glycine site on NMDA receptors, D-serine and ALX-5407, display similar beneficial effects to clozapine in mouse models of schizophrenia

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Contributions to project

T. Lipina and V. Labrie equally contributed to all experiments and the writing of the manuscript. I. Weiner helped review the manuscript. J.C. Roder supervised the experiments.
Summary

Schizophrenia is characterized by disturbances in sensorimotor gating and attentional processes, which can be measured by prepulse inhibition (PPI) and latent inhibition (LI), respectively. Research has implicated dysfunction of neurotransmission at the NMDA-type glutamate receptor in this disorder. This study was conducted to examine whether compounds that enhance NMDA receptor (NMDAR) activity via the glycine site, D-serine and ALX-5407 (glycine transporter 1 inhibitor), alter PPI and LI in the presence or absence of an NMDAR antagonist, MK-801. C57BL/6J mice were tested in a standard PPI paradigm with three prepulse intensities. LI was measured in a conditioned emotional response procedure by comparing suppression of drinking in response to a noise in mice that previously received 0 (non-preexposed) or 40 noise exposures (preexposed) followed by two or four noise-foot shock pairings. Clozapine (3 mg/kg) and D-serine (600 mg/kg), but not ALX-5407, facilitated PPI. MK-801 dose dependently reduced PPI. The PPI disruptive effect of MK-801 (1 mg/kg) could be reversed by clozapine and ALX-5407, but not by D-serine. All the compounds were able to potentiate LI under conditions that disrupted LI in controls. MK-801 induced abnormal persistence of LI at a dose of 0.15 mg/kg. Clozapine, D-serine, and ALX-5407 were equally able to reverse persistent LI induced by MK-801. D-serine and ALX-5407 display similar effects to clozapine in PPI and LI mouse models, suggesting potential antipsychotic action. Moreover, the finding that agonists of NMDARs and clozapine can restore disrupted LI and disrupt persistent LI may point to a unique ability of the NMDA system to regulate negative and positive symptoms of schizophrenia.
Experimental Overview

We examined whether NMDAR-enhancing drugs could be therapeutic in treating schizophrenia-like symptoms. The effects of the direct NMDAR glycine site agonist D-serine and the high affinity glycine transporter 1 (GlyT-1) inhibitor ALX-5407 were examined in behavioral animal models relevant to schizophrenia using C57BL/6J mice. The effects of D-serine and ALX-5407 were also compared to those of a classical atypical antipsychotic, clozapine. The animal models employed were PPI and LI. Animal models are an important means for testing novel pharmacological strategies, and PPI and LI are the two paradigms currently dominating neuropharmacological research of schizophrenia.

PPI is a measure of sensorimotor gating, and refers to the attenuation of the startle response by a weak stimulus (prepulse) appearing a short time prior to the presentation of the startle stimulus287. PPI is highly conserved among vertebrates, and is one of the few paradigms in which humans and rodents are tested in similar fashions288. Abnormalities in PPI are not unique to a single pathology, and have been found in patients with obsessive-compulsive disorder, Huntington’s disease, attentional deficit disorder, and Tourette’s syndrome289. Deficits in PPI have been reported in schizophrenia, since an impaired ability to filter or ‘gate out’ sensory information is a prominent clinical feature of this disorder288, 290, and the degree to which PPI is affected, correlates with severity of schizophrenic symptoms288, 291. In rodents, PPI is disrupted by direct and indirect dopamine agonists292-294 as well as by NMDAR antagonists229, 232, 282, 294, and antagonism of the PPI-disruptive effects is taken as an index of antipsychotic activity. Furthermore, atypical antipsychotics have been shown to be more effective than typical ones in reversing PPI deficits induced by NMDAR antagonists193, 295, 296. Although most studies have focused on the reversal of the PPI
disruption induced by dopaminergic agonists or NMDA antagonists, there are reports of PPI potentiating effects of antipsychotics in rats\(^{204}\) and mice\(^{285, 286}\). To date, limited work has been done with agents enhancing NMDAR function.

LI refers to a process whereby previous repetitive exposure to a stimulus without consequence interferes with the ability to form subsequent associations with that stimulus\(^{297, 298}\) (Figure 3.1). It is considered to be a test of selective attention and cognitive flexibility, and examines the ability of an organism to attend to important information in an environment and ignore irrelevant stimuli\(^{297, 298}\) (Figure 3.1). Like PPI, it is measured similarly in both rodents and humans, and is affected in diseases that involve attentional deterioration\(^{288, 297}\). Disorders in information-processing and attention are prominent and occur early in individuals with schizophrenia\(^{297}\). LI is disrupted or absent in humans and rodents given amphetamine, and in schizophrenics exhibiting acute positive symptoms\(^{297-299}\). Consequently, disrupted LI is considered to model positive symptoms of schizophrenia\(^{297, 298, 300}\). Both typical and atypical antipsychotics reverse amphetamine-induced disruption of LI, and reliably potentiate LI under conditions that normally do not yield robust LI\(^{297, 298, 300}\). Unlike amphetamine, acute administration of NMDA receptor antagonists was initially reported to spare LI\(^{301}\). However, Gaisler-Salomon and Weiner\(^{192}\) showed that NMDAR antagonism actually induces abnormally persistent LI, which is demonstrated under conditions that do not yield LI in controls. As found with other NMDAR antagonist-based models, persistent LI was reversed by clozapine and risperidone, but not by haloperidol\(^{192}\). Perseverative behaviors are associated with the negative symptoms of schizophrenia\(^{302, 303}\) and persistent LI correlates with the severity of negative symptoms\(^{299, 304}\). Thus, LI is proposed to model both the
positive (disrupted LI) and negative (persistent LI) symptoms of schizophrenia, and involves the cognitive abilities affected in schizophrenia\textsuperscript{297, 298}.

Deficits in the processing of incoming information are thought to lead to sensory overload, cognitive fragmentation, and thought disorders\textsuperscript{288, 305}. Though PPI and LI are not specific to only the study of schizophrenia, they are the most widely used approaches in modeling the information-processing deficits of schizophrenia with a reasonable amount of face, predictive, and construct validity. Neuroimaging and neuropathological studies have demonstrated that several brain areas found to be abnormal in schizophrenics correspond to areas involved in the sensorimotor gating and LI circuitry, including the hippocampus, nucleus accumbens, and thalamus\textsuperscript{289}. Neurotransmitters that disrupt PPI and LI, such as dopamine, equally result in psychotic symptoms relevant to schizophrenia\textsuperscript{297}. Moreover, as in schizophrenia, treatments with antipsychotic efficacy are able to ameliorate impaired PPI and LI\textsuperscript{294, 298}. This is a major advantage that PPI and LI models have over other behavioral tests measuring schizophrenia-like behaviors, such as increased locomotor activity and altered exploratory behaviors. Treatments that have no antipsychotic efficacy, such as antidepressants, are not effective in these paradigms, whereas non-antipsychotic treatments have been successful in reversing abnormal function in other behavioral tests\textsuperscript{294, 298, 306}. Thus, PPI and LI are highly useful approaches that are relevant to the symptoms, brain areas, neurotransmitters, and treatments involved in schizophrenia.

To assess the effects of NMDAR glycine site modulators, D-serine and ALX-5407, on the PPI and LI of C57BL/6J mice, these drugs were administered under various conditions. In both models, we tested: (1) effects of D-serine, ALX-5407, and clozapine on low levels of PPI and LI, in order to determine whether these drugs would potentiate PPI and
LI. Basal PPI levels are relatively low in the C57BL/6J strain\textsuperscript{286} and reduced LI (in controls) was attained by appropriate parametric manipulation; (2) effects of the NMDAR antagonist MK-801 on PPI and LI; and (3) the capacity of D-serine, ALX-5407, and clozapine to reverse MK-801-induced PPI and LI abnormalities (disrupted PPI and persistent LI, respectively).
**Figure 3.1. LI procedure.** LI is a phenomenon whereby previous exposure to a stimulus without consequence prevents subsequent learning of a new association with that stimulus. LI involves a preexposure phase whereby half the animals receive several exposures to an inconsequential tone (PE) and the other half of the animals do not receive tone exposure (NPE). Afterwards all the animals are given a conditioning phase that involves tone-shock pairings. LI is expressed if the animals that received no previous tone exposure (NPE) associate the tone with the shock, and if the animals that did have prior tone exposures (PE) maintain that the tone is meaningless.
Results

Effects of clozapine, D-serine and ALX-5407 on PPI and startle amplitude

PPI at three prepulse intensities (69, 73 and 81 dB) and startle amplitude to a stimulus without a preceding prepulse was examined in C57BL/6J mice after administration of clozapine (3 and 6 mg/kg), D-serine (300, 600, and 900 mg/kg), L-serine (600 mg/kg), and ALX-5407 (1, 10 and 15 mg/kg) (Table 3.1).

Analysis of PPI in animals treated with clozapine revealed a significant main effect of prepulse intensity (F_{2,42} = 11.9, p < 0.001) and drug treatment (F_{2,21} = 6.0, p < 0.01), as well as their interaction (F_{4,42} = 4.5, p < 0.01). Further comparisons indicated a significant main effect of clozapine treatment at 69 dB (F_{2,21} = 6.2, p < 0.01) and at 81 dB prepulse (F_{2,21} = 4.1, p < 0.05), due to the facilitatory effects of the 3 mg/kg dose on PPI at these intensities (p < 0.05; Table 3.1). Clozapine also had a significant effect on startle reactivity (F_{2,21} = 35.8, p < 0.001) at 6 mg/kg (p < 0.001), but not at the 3 mg/kg dose (Table 3.1).

For the D-serine-treated animals, analysis of PPI yielded a significant main effect of prepulse intensity (F_{2,56} = 5.9, p < 0.01), and a main effect of drug which approached significance (F_{3,28} = 2.5, p = 0.055). There was a main effect of D-serine at the 69 dB prepulse intensity (F_{3,28} = 2.6, p ≤ 0.05), as the 600 mg/kg dose of D-serine augmented PPI at this prepulse intensity (p < 0.05; Table 3.1). In contrast, 600 mg/kg of L-serine did not produce any enhancements in PPI, and only a main effect of prepulse intensity was found (F_{2,26} = 6.5, p < 0.01). Additionally, D- and L-serine did not alter the startle amplitude (Table 3.1).
PPI analysis in mice treated with ALX-5407 indicated a significant main effect of prepulse intensity ($F_{2,54} = 4.9, p < 0.01$) and drug treatment ($F_{3,27} = 2.9, p = 0.055$). Further comparisons revealed that 1 mg/kg of ALX-5407 did not alter PPI (Table 3.1), though a diminutive effect at the 10 mg/kg dose was observed at all three prepulse intensities ($p < 0.05$). At 15 mg/kg, ALX-5407 also reduced PPI at the 73 dB and 81 dB prepulse intensities ($p < 0.05$). ALX-5407 did not affect startle reactivity ($F_{3,27} = 1.3, p > 0.05$), however there was a tendency to increase startle amplitude at the highest dose (15 mg/kg) compared to controls ($p = 0.08$; Table 3.1).

**Table 3.1. Effects of clozapine, D-serine, L-serine and ALX-5407 on PPI at three prepulse intensities and startle response**

<table>
<thead>
<tr>
<th></th>
<th>PPI 69 dB (%)</th>
<th>PPI 73 dB (%)</th>
<th>PPI 81 dB (%)</th>
<th>Startle Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clozapine (mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>54.8 ± 6.2</td>
<td>57.6 ± 6.5</td>
<td>59.6 ± 5.2</td>
<td>1213.8 ± 130.1</td>
</tr>
<tr>
<td>3.0</td>
<td><strong>76.3 ± 3.4</strong></td>
<td>70.7 ± 3.5</td>
<td><strong>75.5 ± 3.8</strong></td>
<td>1026.5 ± 60.3</td>
</tr>
<tr>
<td>6.0</td>
<td>51.6 ± 6.7</td>
<td>66.9 ± 8.3</td>
<td>77.8 ± 5.3</td>
<td>238.6 ± 45.0***</td>
</tr>
<tr>
<td><strong>D-serine (mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>45.2 ± 4.8</td>
<td>52.2 ± 6.2</td>
<td>60.9 ± 4.6</td>
<td>1326.1 ± 130.4</td>
</tr>
<tr>
<td>300</td>
<td>47.0 ± 12.8</td>
<td>54.2 ± 13.6</td>
<td>59.7 ± 10.6</td>
<td>1380.2 ± 89.3</td>
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<tr>
<td>600</td>
<td><strong>66.2 ± 3.4</strong></td>
<td>64.0 ± 4.4</td>
<td>70.5 ± 4.0</td>
<td>1123.3 ± 131.7</td>
</tr>
<tr>
<td>900</td>
<td>43.8 ± 9.2</td>
<td>52.4 ± 8.1</td>
<td>51.3 ± 9.2</td>
<td>1414.4 ± 202.3</td>
</tr>
<tr>
<td><strong>L-serine (mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>46.8 ± 5.2</td>
<td>50.4 ± 4.3</td>
<td>52.6 ± 5.2</td>
<td>1257.3 ± 105.4</td>
</tr>
<tr>
<td>600</td>
<td>45.9 ± 8.9</td>
<td>54.5 ± 3.2</td>
<td>55.8 ± 5.9</td>
<td>1414.4 ± 116.5</td>
</tr>
<tr>
<td><strong>ALX-5407 (mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>59.4 ± 3.3</td>
<td>64.4 ± 3.8</td>
<td>68.4 ± 2.9</td>
<td>1245.7 ± 78.2</td>
</tr>
<tr>
<td>1</td>
<td>51.1 ± 7.7</td>
<td>56.2 ± 5.5</td>
<td>64.6 ± 6.2</td>
<td>1292.0 ± 165.8</td>
</tr>
<tr>
<td>10</td>
<td><strong>41.0 ± 7.8</strong></td>
<td><strong>40.3 ± 10.3</strong></td>
<td><strong>44.5 ± 11.6</strong></td>
<td>1455.6 ± 130.3</td>
</tr>
<tr>
<td>15</td>
<td>45.9 ± 6.0</td>
<td>37.6 ± 9.2</td>
<td>44.2 ± 7.9</td>
<td>1561.2 ± 130.4</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. n = 7-8 per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle-treated mice (post-hoc LSD test, ANOVA).
Blockade of the NMDAR with MK-801 disrupts PPI

Analysis of PPI in mice injected with MK-801 revealed a main effect of prepulse intensity ($F_{2,70} = 45.7, p < 0.001$) and drug treatment ($F_{4,35} = 7.4, p < 0.001$), as well as a significant prepulse intensity $\times$ drug treatment interaction ($F_{8,70} = 2.3, p < 0.05$). MK-801 at all doses inhibited PPI at all three prepulse intensities (main effect of drug treatment at 69 dB: $F_{4,35} = 7.8, p < 0.001$, at 73 dB: $F_{4,35} = 5.9, p < 0.001$, and at 81 dB: $F_{4,35} = 4.7, p < 0.01$). Further comparisons revealed a less pronounced effect of MK-801 at the 0.15 mg/kg dose ($p < 0.05$ at all three pre-pulses) and at the 0.3 mg/kg dose ($p < 0.05$ at 69 dB; Figure 3.2). More pronounced effects were found at the 0.6 mg/kg dose on the three prepulse intensities ($p < 0.01$; Figure 3.2). Maximal MK-801 effects were observed at a 1 mg/kg dose on all three prepulse intensities ($p < 0.01$; Figure 3.2). In absence of a prepulse, MK-801 did not alter startle reactivity, though there was a trend toward an increased startle amplitude at the highest dose (1 mg/kg, $p = 0.06$; Table 3.2).
Figure 3.2. NMDAR inhibition disrupts PPI. The effects of MK-801 (0.15, 0.3, 0.6, 1.0 mg/kg) on PPI were examined using three prepulse intensities (69, 73, 81 dB). Data are shown as mean ± SEM. n = 8 per group; *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with vehicle-treated mice.
### Table 3.2. Effects of MK-801, clozapine + MK-801, D-serine + MK-801 and ALX-5407 + MK-801 on startle response

<table>
<thead>
<tr>
<th></th>
<th>Startle Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1218.4 ± 124.8</td>
</tr>
<tr>
<td>0.15 mg/kg MK-801</td>
<td>1344.1 ± 174.6</td>
</tr>
<tr>
<td>0.3 mg/kg MK-801</td>
<td>1389.6 ± 168.4</td>
</tr>
<tr>
<td>0.6 mg/kg MK-801</td>
<td>1150.2 ± 177.0</td>
</tr>
<tr>
<td>1.0 mg/kg MK-801</td>
<td>1568.5 ± 85.9</td>
</tr>
<tr>
<td>Vehicle + Vehicle</td>
<td>1259.9 ± 52.8</td>
</tr>
<tr>
<td>Vehicle + 1.0 mg/kg MK-801</td>
<td>1584.8 ± 171.4*</td>
</tr>
<tr>
<td>Clozapine + MK-801</td>
<td>1117.1 ± 105.5</td>
</tr>
<tr>
<td>D-serine + MK-801</td>
<td>1288.2 ± 114.0</td>
</tr>
<tr>
<td>ALX-5407 + MK-801</td>
<td>1302.1 ± 174.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. 
\(n = 7-10\) per group; \(^*p < 0.05\) in comparison with vehicle-treated mice \((post-hoc \text{ LSD test, ANOVA})\).
Effects of clozapine, D-serine, and ALX-5407 on disrupted PPI induced by MK-801

The capacity of clozapine (3mg/kg), D-serine (600 mg/kg), and ALX-5407 (1 mg/kg) to reverse the PPI deficit induced by MK-801 (1 mg/kg) was examined (Figure 3.3). Administration of clozapine potentiated PPI in vehicle-treated mice and normalized disrupted PPI in animals given MK-801. There was a significant main effect of pretreatment ($F_{1,28} = 8.5, p < 0.01$) and drug treatment ($F_{1,28} = 32.9, p < 0.001$). Mice treated with clozapine + vehicle had elevated PPI compared to mice given only vehicle treatments at the prepulse intensities of 69 and 73 dB ($p < 0.05$; Figure 3.3A). PPI in animals administered clozapine + MK-801 was increased at 69 and 73 dB ($p < 0.05$) compared to MK-801-treated mice, and was similar to that of vehicle-treated mice (Figure 3.3A).

D-serine enhanced basal levels of PPI, but did not significantly improve the MK-801-induced PPI disruption. There was a main effect of pretreatment ($F_{1,35} = 9.3, p < 0.01$) and drug treatment ($F_{1,35} = 64.3, p < 0.001$). Compared to vehicle-treated mice, animals given D-serine + vehicle treatment had significantly increased PPI at all three prepulse intensities ($p < 0.05$), while animals injected with D-serine + MK-801 displayed a reduction in PPI at 69 and 81 dB ($p < 0.01$; Figure 3.3B).

ALX-5407 reversed the MK-801-induced PPI deficit without affecting basal levels of PPI. There was a main effect of drug treatment ($F_{1,28} = 12.7, p \leq 0.001$), a pretreatment × drug treatment interaction ($F_{1,28} = 9.2, p < 0.01$), and a pretreatment × drug treatment × prepulse intensity interaction ($F_{2,56} = 5.2, p < 0.01$). In MK-801-treated mice, disrupted PPI was ameliorated animals given ALX-5407 treatment at the prepulse intensities of 69 dB and 73 dB ($p < 0.01$; Figure 3.3C).
Startle response to a stimulus without a prepulse was evaluated for the five drug conditions: vehicle + vehicle, vehicle + MK-801, clozapine + MK-801, D-serine + MK-801 and ALX-5407 + MK-801 (Table 3.2). Analysis was completed for each experiment examining reversal of the MK-801-induced PPI disruption (Figure 3.3A, 3.3B, and 3.3C). No effects on the startle amplitude were observed, except for in experiment 3.2A ($F_{1,28} = 5.34, p < 0.05$), where MK-801 (1 mg/kg) treatment significantly elevated the startle response ($p < 0.05$; Table 3.2).
A

B

C

% Prepulse Inhibition

Prepulse Intensity
Figure 3.3. Reversal of the effects of an NMDAR antagonist in PPI. The capacity of clozapine (3 mg/kg; cloz) (A), D-serine (600 mg/kg; D-ser) (B), and ALX-5407 (1 mg/kg) (C) to ameliorate an MK-801 (1mg/kg) induced PPI deficit. Three prepulse intensities (69, 73, 81 dB) were used in the PPI assay. Data are presented as mean ± SEM. n = 7-10 per group; **p < 0.01, ***p < 0.001 in comparison with vehicle-treated group; #p < 0.05, ##p < 0.01 in comparison with MK-801-treated mice.
LI with 2 or 4 conditioning trials

LI was evaluated in C57BL/6J mice given 2 or 4 CS-US pairings. On test day, the time taken to complete lick 50-75 (A period) before CS onset did not differ between any group (overall mean A period: 7.9 ± 1.5 s). LI was present in mice given 2 but not 4 CS-US trials (Figure 3.4). There was a main effect of preexposure ($F_{1,29} = 11.6, p < 0.01$) and conditioning trials ($F_{1,29} = 7.9, p < 0.01$), as well as a significant preexposure × conditioning trials interaction ($F_{1,29} = 13.2, p < 0.001$). The suppression ratios between the PE and NPE groups were significantly different in the procedure with 2 conditioning trials ($p \leq 0.01$), but not in the procedure with 4 conditioning trials.

Enhancement of LI by clozapine, D-serine, and ALX-5407

The ability of NMDAR modulators and clozapine to facilitate LI under conditions that disrupt LI in controls was examined. No differences were observed in the time taken to complete 50-75 licks prior to CS onset (overall mean A period: 7.2 ± 1.0 s). Clozapine (3 mg/kg), D-serine (600 mg/kg), and ALX-5407 (1 mg/kg) potentiated LI in mice given 4 CS-US pairings (Figure 3.5). A significant main effect of preexposure ($F_{1,56} = 15.1, p < 0.001$), and drug treatment ($F_{3,56} = 3.2, p < 0.05$) was found. Further analysis indicated the presence of LI in animals treated with clozapine, D-serine, and ALX-5407 ($p < 0.05$), but not in the vehicle-treated animals.
Figure 3.4. LI following 2 or 4 CS-US presentations. Mean suppression ratios of preexposed (PE) and nonpreexposed (NPE) mice conditioned with two (2 CS-US) or four (4 CS-US) noise-shock pairings. PE mice had previously received forty nonreinforced noise preexposures. Data are expressed as mean ± SEM. n = 8-9 per group; **p < 0.001 compared to PE score, within the same CS-US group; ###p < 0.001 compared to PE score with 2 CS-US trials.
Figure 3.5. Clozapine, D-serine, and ALX-5407 potentiate LI. Mean suppression ratios of the preexposed (PE) and nonpreexposed (NPE) mice in four drug conditions: vehicle, 3 mg/kg clozapine, 600 mg/kg D-serine, and 1 mg/kg ALX-5407. Forty noise pre-exposures and four noise-shock pairings were used. Data are presented as mean ± SEM. n = 8 per group; *p < 0.05, **p < 0.01, ***p < 0.001 compared to PE score in the same drug treatment group.
NMDAR antagonism induces abnormally persistent LI

The effects of MK-801 in LI were evaluated in mice given 4 conditioning trials. The time taken to consume 50-75 licks before CS onset was not different in any group (overall mean A period: 6.2 ± 0.8 s). Persistent LI was observed in mice treated with a 0.15 mg/kg dose of MK-801 (Figure 3.6). A significant preexposure × drug interaction (F_{4,68} = 3.5, p ≤ 0.01) was found. LI was present in the 0.15 mg/kg MK-801 condition (p < 0.05), but not in the vehicle, 0.05, 0.1 and 0.2 mg/kg MK-801 conditions.

Clozapine, D-serine, and ALX-5407 reverse persistent LI induced by MK-801

The capacity of NMDAR modulators and clozapine to ameliorate MK-801-induced LI persistence was assessed. No difference between any group were observed in the time taken to complete 50-75 licks prior to the CS (overall mean A period = 9.1 ± 1.2 s). Clozapine (3 mg/kg), D-serine (600 mg/kg), and ALX-5407 (1 mg/kg) each reversed the abnormally persistent LI (Figure 3.7). There was a significant main effect of preexposure (F_{1,69} = 5.3, p < 0.05) and a significant pre-exposure × drug treatment interaction (F_{4,69} = 4.3, p < 0.01). Vehicle-treated mice did not show LI, whereas MK-801-treated mice demonstrated LI (p < 0.001). MK-801-treated animals that received concomitant injections of clozapine, D-serine, and ALX-5407 displayed no LI, similar to vehicle-treated controls.
Figure 3.6. NMDAR inhibition induces persistent LI. Mean suppression ratios of the preexposed (PE) and nonpreexposed (NPE) mice in five drug conditions: vehicle, 0.05 mg/kg MK-801, 0.1 mg/kg MK-801, 0.15 mg/kg MK-801, and 0.2 mg/kg MK-801. Forty noise pre-exposures and four noise-shock pairings were used. Data are shown as mean ± SEM. n = 7-8 per group; *p < 0.05 compared with PE score, within the same MK-801 dose.
Figure 3.7. Reversal of MK-801-induced persistent LI by clozapine, D-serine, and ALX-5407. Mean suppression ratios of the preexposed (PE) and nonpreexposed (NPE) mice in five drug conditions: vehicle, 0.15 mg/kg MK-801, 3 mg/kg clozapine + 0.15 mg/kg MK-801, 600 mg/kg D-serine + 0.15 mg/kg MK-801, and 1 mg/kg ALX-5407 + 0.15 mg/kg MK-801. Forty noise pre-exposures and four noise-shock pairings were used. Data are expressed as mean ± SEM. n = 8 per group; ***p < 0.001 compared to PE score, within the same drug treatment group.
Discussion

The main finding of this study was that agents increasing NMDAR function, via effects at the glycine site, potentiated PPI and LI on their own and reversed PPI and LI abnormalities (disrupted PPI and abnormally persistent LI) induced by the NMDA antagonist MK-801, in C57BL/6J mice. Given the great expansion of genetically altered mice and the increasingly central position they occupy in the development of animal models of psychopathology, it is imperative to extend our knowledge of the behaviors of the parental strains used in the creation of these mutant animals. C57BL/6J mice are commonly used in this capacity, and were chosen for this study because the literature and our pilot investigations indicated that this strain shows moderate level of PPI, as well as manifests LI. Since the effects of the NMDAR glycine site modulators were also produced by the atypical neuroleptic, clozapine, these results support the notion that NMDAR glycine site modulators may have an “atypical” antipsychotic profile. Furthermore, the results are consistent with the ability of this class of agents (as well as clozapine) to prevent schizophrenia-like symptoms induced by ketamine in humans, and to ameliorate negative and cognitive symptoms in schizophrenia patients\textsuperscript{167, 175, 183, 307}. Hence, normal and aberrant PPI and LI may provide model systems that could be useful in evaluating treatments of schizophrenia targeting the glutamate receptor.

Prepulse inhibition

Both typical and atypical antipsychotics have been shown to potentiate PPI in mouse strains with low to medium basal levels of PPI, including C57BL/6J mice\textsuperscript{285, 286}. In the present study, clozapine facilitated PPI in C57BL/6J mice at 3 but not at 6 mg/kg. The lack of effect of the higher dose can be attributed to its reducing effect on startle response. This
pattern is consistent with previous findings that clozapine enhances PPI and decreases the
startle response in C57BL/6J mice\textsuperscript{285, 286}.

D-serine had no effect on startle at all three doses, and exerted a facilitatory effect on
PPI at the 600 mg/kg dose. While the lower dose of D-serine could be a sub-threshold
NMDAR activating dose, the high dose may have been ineffective due to hyperactivation of
regulatory negative feedback systems or increases in degrading enzymes to compensate for
the excessively high levels of D-serine. The effect of D-serine is apparently specific to the
glycine binding site because there are no other known neurotransmitter systems affected by
this amino acid, including strychnine-sensitive inhibitory glycine receptors\textsuperscript{308}. The effect was
specific to the \textit{d} enantiomer of this amino acid as L-serine did not enhance PPI.

In contrast to D-serine and clozapine, ALX-5407 did not affect PPI at the lowest dose
(1 mg/kg), and moreover, at higher doses reduced PPI. These outcomes are at variance with
findings showing that ALX-5407 at 1 and 10 mg/kg doses potentiated PPI in DBA/2J mice\textsuperscript{58},
and could reflect strain differences in the sensitivity to ALX-5407 or in the levels of basal
PPI. As for the higher doses, ALX-5407 apparently acted as an antagonist, producing in the
mice increased basal startle reactivity, extreme hyperactivity and strong stereotypy, similar to
effects seen after the administration of NMDAR antagonists\textsuperscript{229, 309}. This notion is consistent
with a report that high concentrations of the GlyT-1 inhibitor CP-802079 lead to a loss of the
NMDA augmentation that is seen with lower concentrations of CP-802079\textsuperscript{64}. Hence, the
effect of ALX-5407 at high doses could indeed be associated with a reduction of NMDAR
activation.

In line with previous demonstrations of NMDAR antagonist-induced reduction in PPI
in rats\textsuperscript{193, 310} and mice\textsuperscript{229, 232}, MK-801 disrupted PPI in C57BL/6J mice in a dose-dependent
manner. As would be expected from previous studies\textsuperscript{193}, MK-801 induced PPI disruption was reversed by clozapine. The sensitivity of the NMDAR antagonist-induced PPI disruption, or the so-called “PCP-PPI model”, to clozapine and other atypical antipsychotics, is considered a major strength of this model insofar as it is believed that it may reveal information that is relevant to treatment-resistant patients. Surprisingly, in spite of the growing evidence that drugs enhancing glycine binding site function can be beneficial in such schizophrenia patients\textsuperscript{167, 175, 183, 307}, to the best of our knowledge, there have been no studies testing the effects of pro-glycine treatments on NMDAR antagonist–induced PPI disruption, although glycine and the GlyT-1 inhibitor ORG-24598 were shown to reverse a disruption in PPI caused by a neonatal ventral hippocampal lesion\textsuperscript{311}. Our results provide the first demonstration that NMDAR antagonist-induced PPI disruptions can be reversed by agents potentiating NMDAR transmission via effects at the glycine site. Of the two such agents used, the highly selective GlyT-1 inhibitor, ALX-5407 reversed the MK-801 induced disruption of PPI whereas D-serine showed only a trend to normalize the impaired PPI. This is consistent with the expectation and findings that increasing synaptic glycine levels by inhibition of glycine reuptake systems should produce a more significant potentiation of NMDAR-mediated neurotransmission than is possible to obtain with exogenous glycine/D-serine/administration\textsuperscript{312}. However, it should be noted that the same dose of ALX-5407 failed to potentiate PPI when given on its own. Thus, it is possible that the effect of increased synaptic levels of glycine differ depending on channel activation state of the NMDA receptors.
Latent Inhibition

In this study, we demonstrated that intact C57BL/6J mice that received 40 noise pre-exposures and 2 noise-shock conditioning trials, showed LI, but when the same number of preexposures was followed by 4 conditioning trials, LI was disrupted. This mimics the reported disruption of LI in rats by extended conditioning in the same Conditioned Emotional Response procedure. Antipsychotics on their own have been repeatedly shown to enhance LI in rats under parametric conditions that reduce or disrupt LI in controls. This effect is specific and selective for drugs with known antipsychotic activity and is not produced by a wide range of non-antipsychotic drugs. Consistent with this body of literature, the present study demonstrated that clozapine potentiates LI in mice, and shows for the first time that the same effect is produced by D-serine and ALX-5407. This behavioral similarity suggests that agents enhancing NMDAR glycine site function may share common features with antipsychotics.

While drug-induced disrupted LI in the rat is considered to model positive symptoms of schizophrenia, LI can exhibit an opposite pole of abnormality, whereby it persists under conditions that normally disrupt LI, and it was suggested that persistence of LI may reflect impaired set shifting, that is associated with cognitive inflexibility and negative symptoms. Excessive LI has been demonstrated in schizophrenia patients, and, most importantly, has been shown to positively correlate with the level of negative symptoms. Gaisler-Salomon and Weiner provided the first results showing that systemic administration of MK-801 in at low dose induces abnormally persistent LI, which can be reversed by the atypical antipsychotic drug clozapine, but not by the typical antipsychotic haloperidol. In line with this finding, we showed that under the LI disrupting parameters of
extended conditioning, C57BL/6J mice treated with a low (0.15 mg/kg) dose of MK-801 have persistent LI. The two lower doses were ineffective, whereas the highest dose impaired conditioning. A similar narrow dose-response curve was observed by Gaisler-Salomon and Weiner\textsuperscript{192}, although in their study, persistent LI was obtained with 0.05 mg/kg. Due to the well-documented propensity of NMDA antagonists to impair or abolish associative learning\textsuperscript{318}, low doses of MK-801 that do not impair conditioning in the non-preexposed rats are imperative for producing persistent LI. This is because the emergence of the LI effect, namely, poorer conditioning of the preexposed compared to non-preexposed rats, is only possible if the drug does not impair conditioning in the non-preexposed group. Moreover, it can be seen in Figures 3.6 and 3.7 that MK-801 affected the non-preexposed and the preexposed groups in an opposite manner, decreasing conditioning in the PE group but increasing conditioning in the NPE group. This distinct effect of a low MK-801 dose on conditioning to a new stimulus and to a stimulus with which the animal had previous experience, is similar to the findings of van der Meulen et al.\textsuperscript{231} which show that MK-801 disrupted discrimination reversal learning without impairing discrimination learning, and that the selective effect of MK-801 on reversal learning was obtained with low doses (0.025 and 0.05) but not with a higher (0.1 mg/kg) dose.

MK-801-induced persistent LI in mice was reversed by clozapine, as well as by D-serine and ALX-5407, such that MK-801-treated animals given these drugs showed no LI, as did vehicle-treated mice. Thus, data obtained from LI experiments demonstrate that direct and indirect modulators of the NMDAR glycine site, as well as clozapine, are able to regulate the two poles of abnormality in LI, which may be associated with positive and negative symptoms of schizophrenia.
**PPI and LI: commonalities and differences**

Glycinergic agents acted more consistently in LI as both potentiated LI and reversed MK-801-induced persistent LI. However, in the case of PPI, potentiation was produced by D-serine but not ALX-5407, and vice versa for MK-801 induced disrupted PPI. The lower dose of MK-801 used in LI compared to PPI, may explain why in the case of LI, D-serine was effective in reversing MK-801 effects. As for potentiation, the robustness of this effect clearly depends on the basal level of the assessed behavior in the controls. While control C57BL/6J mice showed a medium basal level of PPI, the controls in the LI experiments showed no LI at all, because of parametric manipulation to ensure its loss.

Although clozapine affects multiple receptors\(^1\), reversal of NMDAR antagonist induced PPI disruption by clozapine has been attributed to its antagonistic action at the 5-HT\(_2\) receptor, because the same effect is produced by selective 5HT\(_{2A}\) antagonists\(^193\). The same has been argued for clozapine’s ability to reverse MK-801-induced persistent LI\(^192\). If this is the case, then the interaction between clozapine and NMDAR antagonists with regard to their effects on PPI and LI is not due to a competition for a common receptor because NMDAR antagonists do not have an appreciable affinity for 5HT\(_{2A}\) receptors. In contrast, D-serine and ALX-5407 certainly do act to produce their behavioral effects on LI and PPI via activation of the NMDAR. Indeed, both target the NMDAR glycine binding site\(^308,319\).

It remains to be elucidated how NMDAR glycine site stimulation interferes with the action of MK-801. Glycine site stimulation increases the frequency and duration of NMDAR channel opening\(^320,321\), and in this manner may promote the dissociation of MK-801 from the receptor pore\(^321,322\). Alternatively, glycine site stimulation may recruit and activate a larger
proportion of NMDA receptors\textsuperscript{323}, thus "diluting" the effects of NMDAR blockade by MK-801. It should be noted that NMDAR glycine site stimulation could also be expected to enhance MK-801 effects due to an increased ability of MK-801 to reach the open channel site. Indeed, increased MK-801/PCP binding and MK-801 induced locomotor activity following treatment with GlyT-1 inhibitors has been reported\textsuperscript{324,325}. The fact that MK-801 produced here behavioral effects when given on its own indicates that a sufficient proportion of channels were stimulated (in the open state) to allow MK-801 to block the channels. Under such conditions, treatments that induce NMDAR glycine site activation may serve to release the bound antagonist. Whether the opening of NMDA channel caused by glycine site stimulation promotes the “invasion” of MK-801 or its “ejection” is likely to depend on many factors, including the doses of agonists and antagonists employed and possibly the behavior assessed. Moreover, since MK-801 blocks the NMDAR, but also induces glutamate release resulting in activation of other glutamate receptors (AMPA and kainate)\textsuperscript{326}, its behavioral effects can be ultimately due to a deficit or an excess of glutamatergic transmission. Our results indicate that MK-801 was able to induce persistent LI and disrupted PPI due to a deficit in NMDAR transmission. It was shown that ketamine-induced disrupted PPI was reversed by lamotrigine\textsuperscript{327}, which decreases glutamate release, indicating that the ketamine-induced effect is mediated at least in part by excessive glutamatergic transmission. It remains to be investigated whether the effects of MK-801 on PPI and LI are in part also mediated by excessive glutamatergic transmission, as has been found for ketamine-induced psychosis in humans\textsuperscript{328}.

Finally, in recent years, attention has been directed to the ability of clozapine to act as a partial agonist at the NMDAR glycine site\textsuperscript{189,329}, and this mechanism has been suggested to
underpin its unique actions in the clinic and in animal models\textsuperscript{330}. The present findings that clozapine effects are mimicked by pro-glycine treatments, supports this possibility; however, this remains to be investigated.

Although PPI and LI appear to be modulated in a similar manner, the relationship between the drug-induced potentiation and reversal of MK-801 induced aberration is different in PPI and LI. Potentiation of PPI and reversal of MK-801 induced PPI disruption reflect a common process, namely, in both cases, the drugs \textit{strengthen} PPI. The ability of clozapine and glycine binding site agonists to reverse pharmacologic disruption of PPI may be at least partly accounted for by their capacity to potentiate PPI, and both phenomena are likely to be subserved by common mechanisms. This is not the case with LI. Here the drugs \textit{potentiate} LI when given alone but \textit{disrupt} LI when given with MK-801. Therefore, the effects on LI exerted by these drugs on their own and in conjunction with MK-801, likely reflect interactions with distinct neural pathways or systems within the complex forebrain circuitry that regulates LI\textsuperscript{298}. In other words, LI potentiation produced by clozapine and NMDA agonists may depend on actions within brain sites that differ from the sites at which these drugs disrupt MK-801 induced LI. If persistent LI is confirmed to be a behavioral aberration that models well schizophrenia-like pathology, then persistent LI in MK-801-treated mice might be a sensitive approach to uncover novel mechanisms of antipsychotic action.

\textbf{Conclusion}

The suggestion that potentiation of NMDAR function may be useful for the treatment of schizophrenia is derived from the notion that NMDAR hypofunction may be critically involved in the etiology or pathophysiology associated with this disorder. More specifically,
with regard to the role of glycine, genetic evidence suggests that a polymorphism in a primate-specific gene (G72) may be linked to schizophrenia. The protein coded by G72 positively modulates D-amino acid oxidase, which in turn metabolizes D-serine\(^9\). These findings raise the possibility of a primary deficiency of the NMDAR-dependent glycine system in schizophrenia, supplementing the clinical findings that agonists at the glycine binding site are beneficial in schizophrenia patients\(^{167, 175, 183, 307}\). The present results show the ability of PPI and LI models to detect the effects of such agents, although the sensitivity of these models may vary due to the involvement of different neuronal pathways. Since only single doses were used in most of the experiments and only one mouse strain, the present results should be considered preliminary. However, this study is novel in demonstrating that D-serine and ALX-5407 exert in mouse models of PPI and LI behavioral effects considered relevant to the treatment of schizophrenia, supporting the claim that the NMDAR glycine site may be a target for novel antipsychotics.
CHAPTER 4

Mice with reduced NMDA receptor glycine affinity model some of the negative and cognitive symptoms of schizophrenia

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Contributions to project

V. Labrie conducted all experiments and wrote the manuscript. T. Lipina helped with experimental design and assisted in reviewing the manuscript. M. Pauley-Evers provided the \textit{Grin1}^{D481N} mice. J.C. Roder supervised the experiments.
Summary

Schizophrenic patients demonstrate prominent negative and cognitive symptoms that are poorly responsive to antipsychotic treatment. Abnormal glutamatergic neurotransmission may contribute to these pathophysiological dimensions of schizophrenia. We examined the involvement of the NMDA receptor (NMDAR) glycine site in the modulation of negative and cognitive endophenotypes in mice. Behavioral phenotypes relevant to schizophrenia were assessed in \textit{Grin}\textsubscript{1}^{D481N} mice that have reduced NMDAR glycine affinity. \textit{Grin}\textsubscript{1}^{D481N} mutant mice showed abnormally persistent latent inhibition (LI) that was reversed by two agents that enhance NMDAR glycine site function, D-serine (600mg/kg) and ALX-5407 (1mg/kg), as well as by the classical atypical antipsychotic clozapine (3mg/kg). Similarly, blockade of the NMDAR glycine site with the antagonist L-701,324 (5mg/kg) induced persistent LI in C57BL6/J mice. In a social affiliations task, \textit{Grin}\textsubscript{1}^{D481N} mutant animals showed reduced social approach behaviors that were normalized by D-serine (600mg/kg). During a non-associative spatial object recognition task, mutant mice demonstrated impaired reactivity to a spatial change that was reversible by D-serine (300 and 600mg/kg) and clozapine (0.75mg/kg). In contrast, responses to social novelty and non-spatial change remained unaffected, indicating that the \textit{Grin}\textsubscript{1}^{D481N} mutation induces selective deficits in sociability and spatial discrimination, while leaving intact the ability to react to novelty. Genetic and pharmacologically-induced deficiencies in glycine binding appear to model the impairments in behavioral flexibility, sociability, and spatial recognition related to the negative and cognitive symptoms of schizophrenia. Antipsychotics that target the NMDAR glycine site may be beneficial in treating such psychiatric symptoms.
Experimental overview

This study further explores the behavioral effects of reduced NMDAR glycine site activation and specifically focuses on tasks relevant to the negative and cognitive symptoms of schizophrenia. Negative and cognitive symptoms profoundly affect the quality of life of patients with schizophrenia, and current antipsychotics have limited success in treating these symptom domains. Consequently, significant efforts have been made to ameliorate treatments for schizophrenia, and to further elucidate the underlying biological mechanisms.

Preclinical models can enhance our understanding of schizophrenia pathophysiology and pharmacotherapy. As previously mentioned, LI refers to inhibition of conditioned associations to a stimulus by prior exposure to the stimulus in a non-contingent manner. LI is thought to model the deteriorated information-processing abilities seen in schizophrenia patients. LI is impaired in many animal models of schizophrenia, and can be altered in two ways. There can either be a lack of LI or an abnormal persistence of LI, which is considered to represent the positive and negative poles of schizophrenia, respectively.

In the cluster of negative symptoms of schizophrenia, social dysfunction is an important component, beginning in the pre-morbid stages and continuing on chronically throughout the entire course of the illness. Cognitive disturbances in schizophrenia have a similar pattern of emergence, and deficits in attention, spatial organization, and visuo-spatial abilities have been reported to be correlated with social functioning. In animals, measurements of social encounters have served as a heuristic model for the social deficits observed in schizophrenics, while cognitive visuo-spatial abilities can be investigated using a spatial object recognition procedure that evaluates the reaction to a spatial change. Pharmacological studies in rodents have determined prominent impairments in social
interactions and spatial recognition tasks following transient NMDAR blockade.\textsuperscript{233, 270, 336} However, the early appearance of negative and cognitive symptoms indicates that neurodevelopmental animal models may better represent the etiology of these symptoms than acute pharmacological assays.

The present study makes use of the $Grin1^{D481N}$ mice, a genetic model of chronic and developmentally diminished NMDAR glycine site occupancy. These mice have a 5-fold decrease in NMDAR glycine affinity, due to a point mutation (aspartate to asparagine substitution at codon 481) in their NR1 glycine binding site.\textsuperscript{256} Previously, $Grin1^{D481N}$ mutant mice have shown deficits in long-term potentiation, increased startle reactivity, decreased anxiety, and impairments in long-term spatial learning and memory.\textsuperscript{256, 279} Here, we investigate the behavioral effects of diminished NMDAR glycine site function in tasks related to the negative and cognitive symptoms of schizophrenia. Consequently, latent inhibition, social affiliations, and spatial object recognition were measured in the $Grin1^{D481N}$ mice. In these tasks, the efficacy of D-serine to reverse the abnormal phenotypes was assessed and compared to the conventional atypical antipsychotic, clozapine.

**Results**

$Grin1^{D481N}$ mutant mice display persistent LI that is reversible by D-serine, ALX-5407, and clozapine

LI following 2 or 4 conditioning trials (CS-US) was measured in the $Grin1^{D481N}$ mice. No differences were found in the drinking performance of mutant mice during the pre-training days, as measured by the latency to the first lick (wild type: $135.1 \pm 34.6$ s; mutant:
127.1 ± 34.4 s) and the number of licks (wild type: 526.3 ± 36.7; mutant: 476.5 ± 20.4). On test day, the time taken to complete lick 50-75 (A period) before CS onset did not differ between genotype, drug treatment, or pre-exposure group (2 CS-US group: 6.7 ± 1.8 s, 4 CS-US drug-naïve group: 7.6 ± 1.2 s, 4 CS-US drug-treated group: 5.1 ± 0.5 s). Male and female suppression ratios were combined for each pre-exposure condition, as no gender effects were observed. Two mice were excluded because of a failure to drink on the baseline day (mutant in the 2 CS-US group, mutant in the 4 CS-US drug-treated group).

2 CS-US. LI was present in both wild-type and mutant mice given 2 CS-US (main effect of pre-exposure: F1,28 = 24.5, p < 0.001), as there was a difference between the PE and NPE groups within each genotype (p < 0.01; Figure 4.1A).

4 CS-US. LI was also assessed in wild-type and mutant mice given 4 CS-US pairings (Figure 4.1B). A significant main effect of pre-exposure (F1,32 = 5.9, p < 0.05) was found. In contrast to wild-type animals, \textit{Grin1}^{D481N} mutant mice continued to show LI despite the increase in conditioning trials (p < 0.01). Aberrantly persistent LI in mutant animals could be reversed by the direct NMDAR glycine site agonist D-serine\textsuperscript{44}, the high affinity inhibitor of the glycine transporter type 1 ALX-5407\textsuperscript{319}, and the atypical antipsychotic clozapine (Figure 4.1C). Enduring LI was demonstrated in vehicle-injected \textit{Grin1}^{D481N} mutant mice (p < 0.05). However, in mutant mice given D-serine, ALX-5407, or clozapine, LI was not present, similar to wild-type mice given 4 CS-US pairings. In the ALX-5407 and clozapine groups, the NPE ratio was significantly elevated compared to the NPE ratio of vehicle-treated mutant mice (p < 0.05).
Figure 4.1. Reduced function of the NMDAR glycine site results in persistent LI that is reversible by D-serine, ALX-5407, and clozapine. Mean suppression ratios of pre-exposed (PE) and non pre-exposed (NPE) wild-type (+/+) and Grin1\textsuperscript{D481N} mutant (D481N/D481N) mice given 2 CS-US (A) or 4 CS-US (B) pairings. (C) Mean suppression ratios of mutant mice that received 4 CS-US pairings and treatments of vehicle (veh), D-serine (600mg/kg; D-s), ALX-5407 (1mg/kg; ALX), or clozapine (3mg/kg; cloz). Data are presented as mean ± SEM. n = 7-10 per group; *p < 0.05, **p < 0.01 compared to PE score within each genotype or drug treatment; #p < 0.05, ##p < 0.01 compared to NPE score of vehicle-treated mutant mice.
LI in C57BL/6J mice treated with a NMDAR glycine site antagonist or a NR2B antagonist

To confirm the role of reduced NMDAR glycine site occupancy in persistent LI, an antagonist, L-701,324, with high affinity and selectivity for the NMDAR glycine site, was administered to male C57BL/6J mice. NR2B receptor blockade using a selective antagonist, Ro 25-6981, was also tested to determine whether the involvement of the NR1 subunit in persistent LI could be due to its association with the NR2B subunit. NMDA-NR2B receptors have previously demonstrated a role in cognitive flexibility in rodents\textsuperscript{279, 337, 338}. To assess the effects of the antagonists under conditions that produce LI in vehicle-treated mice, a 2 CS-US protocol was used. To estimate the ability of the drugs to influence persistent LI, a 4 CS-US protocol was applied. No differences were demonstrated between drug treatment or pre-exposure groups in the time taken to consume 50-75 licks prior to CS onset (2 CS-US group: 8.5 ± 1.5 s, 4 CS-US group: 9.7 ± 1.7 s).

2CS-US. Initially, C57BL/6J mice treated with either vehicle, L-701,324, or Ro 25-6981 were given 2 CS-US pairings (Figure 4.2A). There was a significant main effect of pre-exposure (F\textsubscript{1,44} = 28.2, \( p < 0.001 \)) and drug treatment (F\textsubscript{2,44} = 7.3, \( p < 0.01 \)), and LI was present in all treatment groups (\( p < 0.05 \)). Also, animals given Ro 25-6981 had a significantly higher NPE ratio compared to vehicle-treated mice (\( p < 0.05 \)), suggesting that though LI is present in Ro 25-6981-treated mice, this group may also exhibit a minor reduction in associative learning.

4 CS-US. LI was not demonstrated in vehicle-treated C57BL/6J mice given 4 CS-US pairings (Figure 4.2B). However, a main effect of drug treatment (F\textsubscript{2,62} = 8.4, \( p < 0.001 \)) was determined, since LI was present in animals given L-701,324 (\( p < 0.05 \)). The PE score of L-
701,324-treated mice was significantly elevated compared to the PE score of vehicle-treated mice ($p < 0.01$), indicating that the persistence of LI is likely due to a specific alteration in information-processing rather than in learning. Mice given Ro 25-6981 did not display LI, suggesting that NR2B antagonism does not induce persistent LI at a dosage that we previously found capable of producing substantial persistence in C57BL/6J mice in the Morris water maze\textsuperscript{279}. Ro 25-6981-treated mice also had a higher NPE ratio compared to vehicle-treated mice ($p < 0.01$), indicating a decrease in conditional learning.
Figure 4.2. Pharmacological blockade of the NMDAR glycine site, but not of NR2B receptors, induces persistent LI. Mean suppression ratios of pre-exposed (PE) and non pre-exposed (NPE) C57BL/6 mice administered vehicle (veh), L-701,324 (5mg/kg; L-701), or Ro 25-6981 (5mg/kg; Ro). Drug effects were examined in animals given 2 CS-US (A) or 4 CS-US (B) pairings. Data are expressed as mean ± SEM. n = 7-15 per group; *p < 0.05, **p < 0.01, ***p < 0.001 compared to PE score within each drug treatment; #p < 0.05, ##p < 0.01 compared to vehicle-treated C57BL/6J mice with the same pre-exposure condition (PE or NPE).
*Grin1*<sup>D481N</sup> mutant mice have a social approach deficit that is ameliorated by D-serine

To further investigate the role of reduced glycine affinity in negative-like symptoms of schizophrenia, the social approach behaviors of the *Grin1*<sup>D481N</sup> mice were assessed. In a test of sociability, wild-type preferred the chamber containing an unfamiliar conspecific mouse (stranger 1) over the empty chamber, whereas the mutant mice did not (main effect of genotype: F<sub>1,25</sub> = 12.8, *p* < 0.01; Figure 4.3A). Conversely, wild-type and mutant mice performed similarly in the social novelty phase (Figure 4.3B). A main effect of apparatus side (F<sub>1,25</sub> = 40.2, *p* < 0.001) was due to both wild-type and mutant animals (*p* < 0.001) favoring the chamber containing a newly introduced mouse (stranger 2) over the chamber containing a now familiar mouse (stranger 1). Additionally, the exploratory activity of wild-type and mutant mice was similar, as no significant genotype differences in the number of chamber entries were found in either the sociability or social novelty phase (Figure 4.3C and 4.3D). In the social novelty phase there was a significant main effect of apparatus side (F<sub>1,25</sub> = 18.0, *p* < 0.001), as more entries were made into the chamber containing stranger 2 by wild-type and mutant animals (*p* < 0.05).

The selective social approach deficit of *Grin1*<sup>D481N</sup> mutant mice was further confirmed in an experiment involving a second sociability phase (Figure 4.3E). A significant genotype × apparatus side interaction (F<sub>1,14</sub> = 12.9, *p* < 0.01) was found. In session 1 and 2, a preference for the chamber with an unfamiliar mouse (stranger 1 or 2) compared to the empty chamber was observed in wild-type (*p* < 0.05), but not mutant mice. However, when given a choice between a novel (stranger 3) or familiar partner (stranger 2), both wild-type and mutant animals demonstrated a preference for social novelty (*p* < 0.01).

The effectiveness of D-serine to normalize social approach deficits in *Grin1*<sup>D481N</sup>
mutant mice was assessed and compared to clozapine (Figure 4.4A). In contrast to vehicle-treated mutants, D-serine-treated mutant mice spent more time in the chamber with the unfamiliar conspecific than in the opposite empty cage chamber (main effect of drug treatment: $F_{2,54} = 5.7, p < 0.01$, apparatus side: $F_{1,54} = 34.8, p < 0.001$, and genotype × drug treatment × apparatus side interaction: $F_{2,54} = 4.1, p < 0.05$). A significant preference for the chamber containing the stranger mouse was seen in wild-type mice treated with either vehicle, D-serine, or clozapine ($p < 0.05$) and in D-serine-treated mutant mice ($p < 0.05$), but not in mutant animals injected with vehicle ($p > 0.05$) or clozapine (trend $p = 0.06$).

The exploratory activity of vehicle-treated mutant mice did not differ from vehicle-treated wild-type mice, as demonstrated by the number of entries into the stranger and empty side (Figure 4.4B). However, a reduction in the number of entries into each chamber was observed in wild-type and mutant mice injected with D-serine or clozapine. There was a main effect of drug treatment ($F_{2,54} = 16.2, p < 0.001$), and apparatus side ($F_{1,54} = 16.5, p < 0.001$). D-serine- or clozapine-treated mice displayed fewer entries into the stranger and empty side ($p < 0.05$), and mutant mice given D-serine or clozapine favored the side with the stranger mouse ($p < 0.05$).

The deficit in social behavior of the $Grin1^{D481N}$ mutant mice and its amelioration by D-serine could not be explained by changes in olfactory function, as no differences in the latency required to find a buried food pellet were found (wild-type mice treated with vehicle: 143.1 ± 38.6 s, 600mg/kg D-serine: 108.3 ± 29.7 s, 0.75mg/kg clozapine: 112.0 ± 10.7 s; mutant mice treated with vehicle: 75.8 ± 10.3 s, 600mg/kg D-serine: 89.0 ± 23.2 s, 0.75mg/kg clozapine: 95.9 ± 11.3 s).
Figure 4.3. Mice with diminished NMDAR glycine affinity have impaired social approach behaviors. Wild-type (+/+) and Grin1D481N mutants (D481N/D481N) mice were assessed in a social affiliations task. (A) Mean time (sec) spent in a chamber containing a stranger mouse, a central chamber, and a chamber with an empty cage in the sociability session. (B) Mean time (sec) spent in a chamber containing a newly introduced mouse (stranger 2), a central chamber, and a chamber with a familiar mouse (stranger 1) in the social novelty session. Number of chamber entries during the sociability (C) and social novelty (D) session. (E) Mean time (sec) spent with stranger 1 (session 1) or stranger 2 (session 2), a central chamber, and a chamber with an empty cage during the sociability sessions (left panel). Mean time (sec) spent exploring a chamber with a novel mouse (stranger 3), a central chamber, and a chamber with a familiar mouse (stranger 2) during the subsequent social novelty session (session 3; right panel). Data are presented as mean ± SEM. n = 11 (A-D) or 8 (E) wild-type and 16 (A-D) or 8 (E) mutant mice; *p < 0.05, **p < 0.01, ***p < 0.001 compared to the chamber with an empty cage or a familiar mouse, within each genotype and session.
Figure 4.4. Social approach deficits in \textit{Grin1}^{D481N} mutant mice are normalized by administration of D-serine. Wild-type (+/+) and \textit{Grin1}^{D481N} mutants (D481N/D481N) animals injected with vehicle (veh), D-serine (600mg/kg; D-s), or clozapine (0.75mg/kg; cloz) were assessed in a test of sociability. (A) Mean time (sec) spent in a chamber containing a stranger mouse, a central chamber, and a chamber with an empty cage. (B) Number of stranger and empty chamber entries. Data are shown as mean ± SEM. \(n = 8-11\) per group; *\(p < 0.05\), **\(p < 0.01\) compared to the chamber with an empty cage, within genotype and drug treatment group; #\(p < 0.05\), ##\(p < 0.01\), ###\(p < 0.001\) compared to vehicle-treated group within genotype and apparatus side.
Mice with reduced NMDAR function have a spatial recognition impairment that is reversed by D-serine and clozapine

Since cognitive deficits are a core feature of schizophrenia and include impairments in spatial recognition\(^1\)\(^,\)\(^2\), the \textit{Grin1}^{D481N} mice were tested in a spatial object discrimination task. In session 1 (S1), mice were placed into an empty arena and no genotype differences in locomotor activity were detected (wild-type mice: 485.9 ± 45.9 beam breaks; mutant mice: 465.6 ± 28.0 beam breaks). The duration of locomotor activity in the object habituation sessions (S2-S5), and in the subsequent sessions of spatial change (S6), and non-spatial change (S7) also did not differ between wild-type and mutant mice (Figure 4.5A).

Wild-type and mutant mice progressively reduced their exploration of all four objects across habituation sessions similarly (main effect of session: \(F_{3,42} = 12.9, p < 0.001\); Figure 4.5B). The time spent exploring each object was also analyzed and confirmed that there was not a preferential exploration of any object during the habituation sessions (genotype × session × object interaction: \(F_{9,126} = 1.5, p = 0.15\)).

The exploration time of the displaced objects (DO) and non-displaced objects (NDO) was evaluated in the last habituation session (S5) and in the spatial change session (S6) (Figure 4.5C). In the last habituation session (S5), all mice spent a similar amount of time exploring the two object categories (DO and NDO). In session 6, when two objects were displaced, mutant mice demonstrated an inability to selectively react to a spatial change. There was a main effect of genotype (\(F_{1,14} = 10.4, p < 0.01\), object category (\(F_{1,14} = 33.7, p < 0.001\)), and genotype × object category interaction (\(F_{1,14} = 40.3, p < 0.001\)). More time was spent exploring the DO than the NDO by wild-type animals (\(p < 0.001\)), whereas mutant mice explored both object categories for a similar amount of time. Furthermore, comparisons
between S5 and S6 revealed that greater time was spent with the DO in S6 than in S5, by both wild-type and mutant animals ($p < 0.05$). Also, the amount of time spent with the NDO in S6 compared to S5 did not differ in wild-type animals, but was significantly increased in mutant mice ($p < 0.01$). This indicates that in wild-type mice, increased exploration was targeted to the objects that underwent a spatial change, whereas in mutant mice, exploratory activity non-specifically increased for both object categories (DO and NDO).

In session 7, when one of the NDO was substituted, both wild-type and mutant mice responded to the non-spatial change (Figure 4.5D). There was a main effect of object category ($F_{1,14} = 30.0, p < 0.001$) related to the preferential exploration of the novel object (NO) compared to the three familiar objects (FOs) by wild-type and mutant animals ($p < 0.01$). A within object category analysis indicated a similar pattern of object investigation.

The efficacy of D-serine to reverse the spatial recognition impairment in mice with reduced NMDAR function was examined and compared to clozapine. A lower dose of D-serine was investigated in tandem to the 600mg/kg D-serine dosage, to limit the possibility of exploratory changes. Locomotor activity in session 1 did not differ between wild-type and mutant mice treated with either vehicle, D-serine or clozapine (Table 4.1). Duration of locomotor activity in the sessions of object habituation (S2-S5) and spatial change (S6) also did not differ between any group (Figure 4.6A).

Mice in all treatment groups demonstrated a comparable gradual decrease in the exploration time of all four objects during the habituation phase (main effect of session: $F_{3,177} = 75.3, p < 0.001$; Figure 4.6B). Analysis of the time spent investigating each individual object indicated that exploration again did not favor any object (genotype × drug treatment × session × object interaction: $F_{27,531} = 1.2, p = 0.24$).
Deficient reactivity to a spatial change was improved by D-serine and clozapine treatments in *Grin1<sup>D481N</sup>* mutant mice (Figure 4.6C). In the last habituation session (S5), exploration of DO and NDO did not differ between any group. However in the session of spatial change (S6), D-serine- or clozapine-treated mutant mice, but not vehicle-treated mutant animals, reacted by selectively exploring the DO more than the NDO. There was a significant main effect of object category ($F_{1,65} = 131.7, p < 0.001$) and genotype $\times$ drug treatment $\times$ object category interaction ($F_{3,65} = 7.4, p < 0.001$). The exploration time of the DO and NDO differed in vehicle, D-serine, or clozapine-injected wild-type mice ($p < 0.05$) and in mutant mice given D-serine or clozapine ($p < 0.05$), but not in vehicle-injected mutant animals. Comparisons between S5 and S6 indicated that all groups spent more time exploring the DO in S6 than in S5 ($p \leq 0.05$), and that the time spent with the NDO did not differ between sessions, except for in vehicle-treated mutant mice which investigated the NDO more in S6 than in S5 ($p < 0.01$). Also, the visual placing test confirmed that visual acuity was normal in all genotype and drug treatment groups (wild-type mice treated with vehicle n=9, 600mg/kg D-serine n=10, 0.75mg/kg clozapine n=8; mutant mice treated with vehicle n=8, 600mg/kg D-serine n=8, 0.75mg/kg clozapine n=8).
A

Duration of locomotor activity (sec)

B

Object exploration (sec)

C

Session 5

Displaced objects
Non-displaced objects

Session 6 - spatial change

D

Session 7 - non-spatial change

Novel object
Familiar objects
Figure 4.5. The $Grin1^{D481N}$ mutation in mice disrupts the ability to discriminate a spatial change. (A) Mean duration (sec) of locomotor activity in the sessions of habituation (S2-S5), spatial change (S6), and non-spatial change (S7) for wild-type (+/+) and $Grin1^{D481N}$ mutant (D481N/D481N) mice. (B) Mean duration (sec) of object exploration in the habituation sessions (S2-S5). (C) Average time (sec) spent exploring the displaced and non-displaced objects for wild-type and mutant animals during the last habituation session (5; left panel) and the spatial change session (6; right panel). (D) Average time (sec) spent exploring the novel item and three familiar objects in the non-spatial change session (7). Data are presented as mean ± SEM. n = 8 wild-type and 8 mutant mice; **$p < 0.01$, ***$p < 0.001$ compared to the time spent exploring the non-displaced or familiar objects, within each genotype; #p < 0.05, ###$p < 0.001$ compared to the time the wild-type mice spent exploring the same object category.
Table 4.1. Locomotor activity in the drug-treated groups during S1 of the spatial recognition experiment

<table>
<thead>
<tr>
<th></th>
<th>Locomotion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grin1&lt;sup&gt;+/+&lt;/sup&gt;</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>256.6 ± 37.9</td>
</tr>
<tr>
<td>D-serine 300</td>
<td>315.8 ± 28.6</td>
</tr>
<tr>
<td>D-serine 600</td>
<td>313.0 ± 31.7</td>
</tr>
<tr>
<td>clozapine</td>
<td>340.9 ± 32.2</td>
</tr>
<tr>
<td><strong>Grin1&lt;sup&gt;D481N/D481N&lt;/sup&gt;</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>283.4 ± 29.5</td>
</tr>
<tr>
<td>D-serine 300</td>
<td>246.7 ± 29.4</td>
</tr>
<tr>
<td>D-serine 600</td>
<td>320.0 ± 20.0</td>
</tr>
<tr>
<td>clozapine</td>
<td>374.9 ± 69.3</td>
</tr>
</tbody>
</table>

Data are expressed as number of beam breaks ± SEM. n = 7-14 per group.
Figure 4.6. Treatment with D-serine or clozapine ameliorates impaired spatial object discrimination in *Grin1*<sup>D481N</sup> mutant mice. Wild-type (+/+), and *Grin1*<sup>D481N</sup> mutant (D481N/D481N) animals were given either vehicle (veh), D-serine (300 or 600mg/kg; D-s), or clozapine (0.75mg/kg; cloz) treatments and reactivity to a spatial change was examined. 

**A** Mean duration (sec) of locomotor activity in the habituation session (S2-S5), and spatial change session (S6). 

**B** Mean duration (sec) of object exploration in the habituation session (S2-S5). 

**C** Average time (sec) spent exploring the displaced and non-displaced objects during the last habituation session (5; left panel) and the spatial change session (6; right panel). Data are shown as mean ± SEM. n = 7-14 per group; *p < 0.05, **p < 0.01, ***p < 0.001 compared to time spent exploring the non-displaced objects, within genotype and drug treatment group; #p < 0.05, ##p < 0.01 compared to the time the vehicle-treated wild-types spent exploring the displaced objects.
Discussion

In this study, we demonstrated that a reduction in NMDAR glycine affinity in mice induced abnormally persistent latent inhibition, social approach deficits, and impairments in spatial recognition that were all reversed by D-serine treatment. Moreover, pharmacological blockade of the NR1 glycine site, but not of NR2B receptors, also produced perseverative LI in C57BL/6J mice. These findings suggest that diminished NMDAR glycine site occupancy in mice induces behavioral disturbances that resemble some of the negative and cognitive impairments of schizophrenia, and that these may be improved by D-serine.

Compared to studies involving acute challenge with NMDAR antagonists, the advantage of tests examining the effect of genetic NMDAR perturbations is that they model the chronic and presumed developmental nature of NMDAR hypofunction theorized to occur in schizophrenia. Mice that express minimal NR1 levels (5-10%) have demonstrated substantial deficits in PPI, startle habituation, social and sexual interactions, as well as increased motor activity, stereotypy, and sensitivity to amphetamine. Animals carrying point mutations in the NR1 subunit, including the NR1^{+/N598Q} and Grin1^{D481N/K483Q} mice, have shown dysfunctions in maternal nurturing, hyperactivity, enhanced stereotypy, impaired spatial reference memory, and striatal dopaminergic and serotonergic hyperfunction. These studies together with our findings indicate that models involving disturbances in the NMDA-NR1 subunit produce phenotypes that are potentially relevant to schizophrenia symptomatology.

Although genetic linkage studies have not associated polymorphisms in the NMDAR to schizophrenia, there are a number of studies that have implicated genes that specifically modulate the NMDAR glycine binding site. These include D-amino acid oxidase
and G72, that are involved in D-serine catabolism, as well as the D-serine synthesis enzyme, serine racemase. Morita et al. found that a SNP associated with a 60% reduction in serine racemase promoter function was significantly elevated in patients with schizophrenia. Accordingly, serum and CSF levels of D-serine have been shown to be decreased in schizophrenic patients, while endogenous antagonists for the NMDAR glycine site were observed to be increased in the CSF and cortex. Therefore, the decreased occupancy and activation of the NMDAR glycine site in the mice is of relevance to the neural changes proposed to occur in schizophrenic individuals, and may be a more appropriate model for NMDAR hypofunction in schizophrenia than mutations involving a loss of function due to reduced expression of the NR1 subunit.

In this study, we found that LI was normally expressed in mutant animals given 2 CS-US trials. This is in agreement with several LI studies that have demonstrated that low doses of NMDAR antagonists do not abolish LI. However, when experimental conditions were increased to 4 CS-US pairings, LI was disrupted in wild-type mice, but persevered in mutant mice. This finding is the first to demonstrate abnormally persistent LI in a genetic animal model of NMDAR hypofunction. Induction of LI persistence due to reduced NMDAR glycine affinity is further supported by the result that pharmacological inhibition of this site, using L-701,324, produced a similar effect. LI perseveration indicates an impaired switching ability, since unlike the pre-exposed wild-types, mutant and L-701,324-treated mice were not capable of switching from ignoring an irrelevant stimulus to responding to the tone-shock association. Thus, the NMDAR glycine site is permissive for appropriate switching responses. Reduced occupancy of this site may lead to behavioral inflexibility, which is a prominent feature of the negative symptoms of schizophrenia.
LI perseveration was reversed in \textit{Grin1}^{D481N} mutant mice by D-serine, ALX-5407, and clozapine. Previously, we have shown that identical dosages of these compounds can reverse enduring LI induced by MK-801 in C57BL/6J mice\textsuperscript{281}. Since both D-serine and ALX-5407 potentiate the glycine binding site\textsuperscript{44,319}, NMDAR activation is the likely explanation for their capacity to overcome the effects of the \textit{Grin1}^{D481N} mutation. In contrast, the mechanism by which clozapine reverses persistent LI has been proposed to involve antagonism of 5-HT\textsubscript{2A} receptors\textsuperscript{192,298}. It has also been theorized that clozapine may exert its beneficial effects in animal models and in the clinic by enhancing NMDAR function\textsuperscript{12}.

Activity-dependent redistribution of NMDARs may account for the reason glycineric treatments and clozapine normalize persistent LI by slightly augmenting NPE scores in this and other studies\textsuperscript{281,344}. Recently, it has been shown that NMDAR glycine site stimulation promotes the priming of these receptors for clathrin-dependent endocytosis that occurs following their activation\textsuperscript{49}. Consequently, the reversal of persistent LI by compounds promoting NMDAR function may involve receptor internalization, which perhaps weakens nonessential circuits. Additionally, reduced NMDAR activity in hippocampal synapses was found to recruit additional NMDARs to the synapse by lateral diffusion from extrasynaptic sites\textsuperscript{345}, and elevations in NMDAR subunit expression were observed in \textit{Grin1}^{D481N} mutant mice\textsuperscript{256}. Thus, a larger pool of (abnormally functioning) NMDARs may explain why LI perseveration in \textit{Grin1}^{D481N} mutant and MK-801-treated mice partially involves an enhancement in associative learning, as demonstrated by lowered NPE scores\textsuperscript{281,344}.

Mutant mice exhibited prominent social deficits that were specific to the test of sociability, since a normal preference for social novelty was demonstrated. The sociability
phase is an examination of social approach- and avoidance-related motivation, whereas the social novelty phase is an assessment of social memory and the ability to discriminate a socially novel stimulus\(^{265,346}\). The social approach impairment in the mutant mice could not be attributed to diminished exploratory activity as the number of chamber entries did not differ. Similar performance was also found in a test of olfactory acuity, suggesting that anosmia is not responsible for the disruption in social motivation. Previous studies with \textit{Grin1}\(^{D481N}\) mutant mice, as well as with NMDAR antagonists, have reported anxiolytic behaviors\(^{256,347}\). Consequently, generalized anxiety-like responses are not likely to be responsible for the reduction in social behavior in mutant mice.

In this experiment, we found that D-serine was more effective than clozapine in reversing the social approach deficit in \textit{Grin1}\(^{D481N}\) mutant mice. Previous, rodent studies have demonstrated conflicting results regarding the ability of clozapine to ameliorate social deficits induced by noncompetitive NMDAR antagonists\(^{230,233,333,336}\). Furthermore, patient studies do not support a direct improvement of primary negative symptoms by clozapine, and instead indicate that any amelioration of negative symptoms is related to clozapine’s effect on positive, extrapyramidal, and depressive symptoms\(^{331}\).

Preference for social novelty was not perturbed by reduced NMDAR glycine affinity. In accordance, it has been reported that antagonists of the NMDAR glycine site given acutely to rats improve social memory and do not disrupt responses to social novelty\(^{348}\). The specific effect of the \textit{Grin1}\(^{D481N}\) mutation on sociability suggests that distinct neural mechanisms may regulate social motivation behaviors and social recognition memory, and that the NMDAR glycine site may be most involved in the former process.

\textit{Grin1}\(^{D481N}\) mice were examined in a cognitive visuo-spatial task involving spatial
object discrimination that has previously been shown to be sensitive to pharmacological manipulation with compounds acting on the dopaminergic or glutamatergic system\textsuperscript{269, 270}. Additionally, spatial recognition performance in this task has also been found to be impaired by lesions to areas such as the nucleus accumbens, hippocampus, and prefrontal cortex, which are recognized to be important for cognitive function and part of the corticolimbic network implicated in schizophrenia\textsuperscript{349, 350}.

During the habituation phase of this experiment, all mice showed similar levels of locomotor activity and object exploration, indicating comparable acclimatization to environment and object configuration. In the spatial change phase, wild-type but not mutant mice exhibited a strong preference for the objects with a different spatial configuration. Interestingly, the mutant animals reacted to the change by re-exploring both displaced and non-displaced objects, as demonstrated by an increased exploration of both object categories in S6 compared to S5. This effect could be interpreted as the mutant animals being able to detect a general change in their environment, while having a selective deficit in their ability to react to a spatial change. Instead they respond as if the entire situation is new. Accordingly, in session 7 the mutant mice displayed a normal ability to respond to a non-spatial change, further indicating a capability of recognizing novelty. Also, previous pharmacological studies have shown that in the same experiment, a NMDAR antagonist impaired spatial recognition, while leaving intact the ability to respond to non-spatial novelty\textsuperscript{269, 270}. Thus, our findings suggest that the NMDAR glycine site is specifically involved in modulating the ability to encode and/or use spatial information in a behavioral task that does not require explicit reinforcement.
The abnormally persistent LI, social deficit, and impaired ability to detect spatial change in Grin1<sup>D481N</sup> mutant mice supports the contention that these animals display behaviors related to the negative and cognitive symptoms of schizophrenia. The ability of D-serine to normalize these behavioral disturbances implies that this proglycinergic treatment may be beneficial in alleviating such symptoms. Additionally, the Grin1<sup>D481N</sup> mice may serve as a useful preclinical model to test novel therapeutic agents for their ability to ameliorate behavioral perturbations induced by chronically diminished NMDAR glycine site occupancy.
CHAPTER 5

Genetic inactivation of serine racemase produces behavioral phenotypes related to schizophrenia in mice

Submitted as:

Contributions to project

V. Labrie established and genotyped the mouse colony, performed the behavioral and pharmacological studies, assisted in the protein assays, analyzed the data, and wrote the manuscript. R. Fukumura conducted the ENU-induced mutation screen. A. Rastogi performed the SNP analysis. L.J. Fick completed the real-time RT-PCR experiments. W. Wang conducted the Srr activity assay and assisted in the protein assays. P.C. Boutros analyzed the microarray data. J.L. Kennedy provided the human blood samples for the SNP analysis. M.O. Semeralul and F.H. Lee prepared the RNA for the microarray study. G. Rauw conducted the HPLC analysis. E. Weiss assisted with genotyping. G.B. Baker, D.D. Belsham, S.W. Barger, Y. Gondo, A.H.C. Wong, and J.C. Roder supervised the experiments conducted in their laboratories.
Summary

Abnormal NMDA receptor (NMDAR) function has been implicated in the pathophysiology of schizophrenia. D-serine is an important NMDAR modulator, and to elucidate the role of the D-serine synthesis enzyme serine racemase (Srr) in schizophrenia, we identified and characterized mice with an ENU-induced mutation that results in a complete loss of Srr activity and dramatically reduced D-serine levels. Mutant mice displayed behaviors relevant to schizophrenia, including impairments in prepulse inhibition, sociability, and spatial discrimination. Behavioral deficits were exacerbated by an NMDAR antagonist and ameliorated by D-serine or the atypical antipsychotic clozapine. This study demonstrates that aberrant Srr function and diminished D-serine may contribute to schizophrenia pathogenesis, and that D-serine may be a beneficial form of treatment.
Experimental Overview

Deficient glutamatergic neurotransmission mediated by the N-methyl-D-aspartate receptor (NMDAR) may be involved in the pathophysiology of schizophrenia. Noncompetitive antagonists of the NMDAR like phencyclidine elicit schizophrenic-like symptoms in healthy individuals and exacerbate such symptoms in patients. Schizophrenia is known to be influenced by heritable factors and genetic studies have identified a number of susceptibility genes that modulate NMDAR function. These include several genes that regulate the endogenous NMDAR glycine site agonist, D-serine. Growing evidence indicates that the D-serine catabolic enzyme, D-amino acid oxidase (DAO), and its activator, G72, are associated with an increased risk of schizophrenia, and DAO activity is augmented in the brains of schizophrenic patients. D-serine is synthesized from L-serine by the enzyme serine racemase (Srr), and recently, a genetic variant associated with 60% reduction in Srr promoter function was found to be significantly more common in patients with schizophrenia. Accordingly, reduced levels of D-serine in the CSF and serum of schizophrenic patients, with a corresponding increase in its precursor, L-serine. Together, these studies indicate that an abnormality in available D-serine may be involved in schizophrenia pathogenesis and prompt a need to further investigate the role of D-serine modulators in schizophrenia.

We examined the role of Srr in schizophrenia by screening mice for a mutation in the Srr gene, and then examined alterations in biochemical markers, behavioral phenotypes, and pharmacological responses. Human and mouse Srr have highly conserved enzymological characteristics, and the use of a mouse model permits an in-depth analysis of the effects
specifically related to reduced Srr function. Here, we report that lack of Srr activity and decreased D-serine levels produce schizophrenia-related phenotypes in mice.

Results

Identification of a mutation in the Srr gene

To establish the Srr mutation in mice, we employed N-nitroso-N-ethylurea (ENU) mutagenesis, an effective means of introducing point mutations into the genome. All Srr isoforms contain coding exons 3, 4, 8, and 9, and these were screened in the F1 progeny of ENU-mutagenized C57BL/6Jcl male mice and untreated DBA/2Jcl female mice (mutation discovery rate of $7.22 \times 10^{-7}$/bp/gamete). At the start of exon 9, a T to A transversion resulting in a nonsense mutation (Tyr269 converted to a stop codon) was identified (Figure 5.1). Exon 9 is the last coding exon of Srr. The C57BL/6Jcl and DBA/2Jcl parental mouse strains have identical exon 9 sequences, indicating that the mutation was induced by ENU treatment. The F1 founder carrying the $Srr^{Y269*}$ mutation was bred with C57BL/6Jcl female mice for one generation (N2), before heterozygous N2 mice were backcrossed for six generations onto the C57BL/6J strain (N3-N8) using male and female animals. Experimental animals were then bred from heterozygous intercrosses of N8 $Srr^{Y269*}$ mice, which contained >99.6% of the C57BL/6J genome. Mutant mice are available from the RIKEN BioResource Center (Rgsc1872; www.brc.riken.jp/lab/animal/en/gscmouse.shtml).
### A

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Figure 5.1. A nonsense mutation in exon 9 of mouse Srr. (A) DNA sequence chromatograms depicting the mutation in Srr. Near the start of exon 9, a transversion (A → T) occurred, which converted amino acid 269 from a tyrosine (TAA) to a stop codon (TAT). DNA sequences for both wild-type (+/+ ) and homozygous mutant mice (Y269*/Y269*) are shown for comparison. (B) Mouse and human Srr protein sequences were aligned using the ClustalX2 program for homology comparison. The human Srr protein shares 89% sequence homology with the mouse protein. Identical residues are marked in gray. The position of the SrrY269* mutation in mice is indicated with red text and an arrow. A line indicates a gap in the alignment, and the blue and black text distinguishes the exons.
Lack of Srr activity and diminished D-serine in mutant mice

The effects of the $Srr^{Y269*}$ mutation on the protein levels and function of Srr were explored in the mouse brain. Western blot analysis showed a loss of Srr protein in the mutant mice using either a monoclonal (Figure 5.2A) or a polyclonal (Figure 5.2B) antibody directed to N-terminal epitopes (main effect of genotype: monoclonal antibody: $F_{2,6} = 140.9, p < 0.001$; polyclonal antibody: $F_{2,6} = 563.4, p < 0.001$). No truncated versions of Srr were observed, based on the predicted size of a putative truncated Srr protein of 29 kDa. Real-time RT-PCR indicated an approximate 50% reduction in $Srr$ mRNA levels in mutant animals (main effect of genotype: $F_{1,10} = 8.0, p < 0.05$; Figure 5.2C). When Srr activity was measured in an assay that examines the production of D-serine above basal levels, no additional D-serine was synthesized in the brain preparations of mutant mice (main effect of genotype: $F_{1,15} = 34.3, p < 0.001$; Figure 5.2D), demonstrating a complete lack of Srr function. Furthermore, D-serine concentrations were greatly reduced in the brain of adult $Srr^{Y269*}$ mutant animals (main effect of genotype: $F_{1,51} = 578.9, p < 0.001$). Lower D-serine levels were found in the whole brain, hippocampus, and frontal cortex ($p < 0.001$), but not in the cerebellum (Figure 5.3). Additionally, in the frontal cortex of mutant mice, an elevation in L-serine levels was observed ($p < 0.01$; Figure 5.3). No changes were detected when the whole brain, hippocampus, frontal cortex, and cerebellum were assayed for the concentrations of other amino acids, including glutamate, glutamine, glycine, arginine, alanine, and GABA (Table 5.1). The expression of other proteins involved in the D-serine pathway and NMDAR-mediated neurotransmission were also evaluated. PICK1 is a scaffolding protein that regulates the synaptic clustering, trafficking to the neuronal surface, and membrane recycling of a several interacting partners$^{143}$. The PDZ domain of PICK1 has
been shown to bind to a consensus sequence on the C-terminus of Srr, possibly to modulate its activity\textsuperscript{106,352}. PICK1 protein levels were elevated in \textit{Srr}\textsuperscript{Y269*} mutant mice (main effect of genotype: F\textsubscript{2,6} = 21.5, \( p < 0.01 \); Figure 5.4A), though mRNA abundance was not significantly increased (Figure 5.4B). In contrast, there was no change in protein levels of the NMDAR-NR1 subunit, AMPA receptor GluR1 and GluR2 subunits, DAO, and glycine transporter-1 (Figure 5.5).
Figure 5.2. Biochemical changes in mice with a nonsense mutation in exon 9 of Srr.

Western blots examining Srr levels were completed using a monoclonal (A1) and a polyclonal (B1) Srr antibody. Protein extracts were from whole brain of wild-type (+/+), heterozygous (+/Y269*), and mutant (Y269*/Y269*) mice. β-Tubulin III was used as a loading control. The molecular weights in kiloDaltons (kDA) of Srr, β-tubulin III, and a protein standard (S) are marked. Densitometric quantification of Western blots conducted with a monoclonal (A2) and polyclonal (B2) Srr antibody. The mean densitometry is an indicator of Srr protein levels in wild-type, heterozygote, and mutant animals. (C) Real-time RT-PCR analysis of Srr mRNA in whole brain of wild-type and mutant mice (n = 6 per group). (D) Srr activity assay. Mean D-serine production (μmol/mg protein/h) by endogenous Srr in wild-type and mutant animals (n = 7-10 per group). Data are shown as mean ± SEM. *p < 0.05, ***p < 0.001 compared to wild-type mice; ##p < 0.01, ###p < 0.001 compared to heterozygous mice.
Figure 5.3. HPLC analysis of L- and D-serine concentrations in Srr\textsuperscript{Y269*} mice. Mean concentration (μg/g tissue) of L-serine (L-s) and D-serine (D-s) were examined in the whole brain, hippocampus, frontal cortex, and cerebellum of wild-type (+/+) and mutant (Y269*/Y269*) mice. Data are presented as mean ± SEM. n = 5-8 per group; **p < 0.01, ***p < 0.001 compared to wild-type mice, within the same brain region and amino acid enantiomer.
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Data are expressed as mean of amino acid concentration (µg/g tissue ± SEM). n = 5-8 per group.
Figure 5.4. Pick1 protein and mRNA levels in SrrY269* mice. (A1) Western blot assessing Pick1 levels. Quantity of Pick1 protein was examined in whole brain tissue of wild-type (+/+), heterozygous (+/Y269*), and mutant (Y269*/Y269*) mice. β-Tubulin III was used as a loading control. The molecular weights in kiloDaltons (kDA) of Pick1, β-tubulin III, and a protein standard (S) are marked. (A2) Densitometric quantification of Western blot for Pick1. The mean densitometry indicates Pick1 protein levels in wild-type, heterozygote, and mutant animals. (B) Real-time RT-PCR analysis of Pick1 mRNA in whole brain of wild-type and mutant mice (n = 6 per group). Data are shown as mean ± SEM. **p < 0.01 compared to wild-type mice; ##p < 0.01 compared to heterozygous mice.
Figure 5.5. Western blots examining proteins that modulate D-serine and NMDAR-mediated neurotransmission. (A1) Protein extracts from whole brain of wild-type (+/+) and mutant (Y269*/Y269*) mice assessed the levels of NR1, GluR1, GluR2, GlyT-1, and DAO. β-Tubulin III was used as a loading control. (A2) Densitometric quantification of Western blots for NR1, GluR1, GluR2, GlyT-1, and DAO. The mean densitometry (± SEM) denotes the quantity of measured proteins in wild-type and mutant mice.
Mutant mice display behavioral phenotypes relevant to schizophrenia

Mice with the Sry^{269*} mutation were physically indistinguishable from their wild-type littermates. Examination of reflexes, vision, fur condition, and weight did not reveal any differences between the genotypes (Figure 5.6A). Performance in a 3-day accelerating rotarod task, an indicator of motor coordination, balance, and motor learning, was similar in wild-type and mutant mice, as comparable latencies to fall from the rotating axle were observed (Figure 5.6B and 5.6C). In an open field arena, locomotor activity throughout the 30-min testing period was not different between wild-type and mutant animals (Figure 5.6D). Additionally, no differences were observed between wild-type and mutant mice in an assessment of depression-like behavior in the forced swim test (Figure 5.6E) or anxiety-like behavior in the elevated plus-maze (Figure 5.6F, 5.6G, and 5.6H).

Social dysfunction is an important component of schizophrenia negative symptoms, often present in the prodromal stages and persisting throughout life^{1,333}. Social behaviors were assessed in Sry^{269*} mice, since these behaviors have served as a model for social deficits in schizophrenia^{333}. During the sociability phase of the social affiliations task, mutant mice displayed a social approach deficit (genotype × apparatus side interaction: F_{1,23} = 6.7, p < 0.05; Figure 5.7A). Wild-type animals preferred the chamber with the unfamiliar conspecific mouse over the empty chamber (p < 0.001), whereas mutant mice did not (Figure 5.7A). In contrast, wild-type and mutant mice demonstrated a comparable performance in the social novelty phase (main effect of apparatus side: F_{1,23} = 26.2, p < 0.001; Figure 5.7B). Both wild-type and mutant animals favored the chamber containing a newly introduced mouse (stranger 2) over the chamber containing a now familiar mouse (stranger 1) (p < 0.01; Figure 5.7B). The social approach deficit observed in mutant mice could not be attributed to
alterations in exploratory activity, as wild-type and mutant animals displayed a similar number of chamber entries (Figure 5.7C and 5.7D). Wild-type and mutant mice had similar olfactory acuity, as both genotypes displayed comparable latencies to find a hidden food pellet (Figure 5.7E).

An impaired ability to filter or ‘gate out’ sensory information is a prominent clinical feature of schizophrenia. A well-established operational measure of sensorimotor gating is prepulse inhibition (PPI) of the startle response. PPI occurs when a low-intensity prepulse precedes a startle stimulus, resulting in a reduction in the startle response. PPI deficits are observed in schizophrenic patients, and PPI is considered to be a model with reasonable face, predictive, and construct validity. mutant mice demonstrated disrupted PPI (main effect of genotype: $F_{1,23} = 6.9, p < 0.05$), as PPI in mutant animals was reduced compared to wild-type animals at the prepulse intensities of 69 dB and 81 dB ($p < 0.05$; Figure 5.8A). In the absence of the prepulse, responses to the startle stimulus (Figure 5.8B) and to a range of stimulus intensities did not differ between genotypes (Figure 5.8C). Also, startle responses were increased at greater stimulus intensities (main effect of stimulus intensity: $F_{10,210} = 24.3$, $p < 0.001$; Figure 5.8C), suggesting normal hearing.

Cognitive deficits are a central characteristic of schizophrenia and include impairments in spatial recognition. In mice, visuo-spatial cognition can be examined using a spatial object recognition procedure. For this task, mice were initially exposed to an empty open field (S1), and locomotor activity during this session did not differ between wild-type and mutant mice (Figure 5.9A). Duration of locomotor activity was also comparable between wild-type and mutant mice in the sessions of object habituation (S2-S4), spatial change (S5), and non-spatial change (S6) (Figure 5.9B). In the habituation sessions
(S2-S4), wild-type and mutant mice showed a similar progressive acclimatization to all four objects (main effect of session: F_{2,34} = 97.0, p < 0.001; Figure 5.9C). Analysis of the time spent investigating each individual object revealed that exploration did not favor any object (genotype × session × object interaction: F_{6,102} = 0.2, p = 1.0). During the last habituation session (S4), the time spent exploring the objects that will be displaced or non-displaced in the subsequent spatial change session demonstrated that wild-type and mutant mice have no bias for either object category (Figure 5.9D).

Assessment of responses in the spatial change session (S5) indicated that mutant mice had a deficit in spatial recognition (Figure 5.9E). There was a significant main effect of object category (F_{1,17} = 65.6, p < 0.001) and a genotype × object category interaction (F_{1,17} = 36.7, p < 0.001). Wild-type mice spent more time investigating the objects with a novel spatial configuration (displaced objects) than the objects that remained stationary (non-displaced objects) (p < 0.001; Figure 5.9E). In contrast, the mutant mice did not react differently to the displaced objects (Figure 5.9E). When a novel object was introduced (S6), both wild-type and mutant animals responded similarly (main effect of object category was found (F_{1,17} = 52.4, p < 0.001) by spending more time exploring the novel object than the three familiar objects (p < 0.01; Figure 5.9F).

Cognitive function in Srr^{Y269*} mice was further evaluated in the Morris water maze (MWM), a classic test of spatial learning and memory. Performance in a visible platform session and in the 7-day acquisition training phase when the platform was hidden was similar in wild-type and mutant mice, as demonstrated by a comparable path length to the target platform (Figure 5.10A). Additionally, the Srr^{Y269*} mutation did not alter sensorimotor abilities or search motivation, as no differences in swim speed, floating, or thigmotaxis
duration were detected between genotypes (main effect of genotype in acquisition trials: swim speed: $F_{1,25} = 0.6, p = 0.4$, floating time: $F_{1,25} = 0.1, p = 0.7$, thigmotaxis time: $F_{1,25} = 0.3, p = 0.6$). However, spatial memory measured in the first probe trial was considerably deficient in $Srr^{y269\ast}$ mutant mice. Compared to wild-type animals, mutant mice spent less time (main effect of genotype: $F_{1,25} = 5.9, p < 0.05$) and made fewer crosses (main effect of genotype: $F_{1,25} = 4.4, p \leq 0.05$) in an area $3\times$ the platform diameter centered over its former location ($p < 0.05$; Figure 5.10B and 5.10C). Furthermore, mutant mice did not display a greater amount of time and crosses over the target location compared to the averaged analogous non-target areas, indicating a lack of preference for the target location (Figure 5.10B and 5.10C). The second probe trial demonstrated that with greater training the spatial memory deficit in mutant mice could be partially ameliorated (main effect of platform location: % time: $F_{1,24} = 17.7, p < 0.001$, crosses: $F_{1,24} = 12.1, p < 0.01$); however, mutant animals in this probe trial continued to lack a significant preference for the target location over the averaged analogous non-target areas (Figure 5.10D and 5.10E).
Figure 5.6. Performance of mutant mice in behavioral measures of motor function, depression, and anxiety. (A) Mean weight (g) of wild-type (+/+) and mutant mice (Y269*/Y269*) at 8, 12, and 18 weeks of age. (B) Mean latencies (sec) to fall from a rotating axle in a 3-day accelerating rotarod procedure containing 3 trials/day. (C) Motor learning in accelerating rotarod procedure. The average motor learning ratio of wild-type and mutant mice demonstrates the ability to improve performance in the accelerating rotarod task across the 3 days. (D) Mean number of beam breaks during a 30-min assessment of locomotion (5-min bins). (E) Forced swim test. Mean time of immobility (sec) of wild-type and mutant mice in a test examining depressive-like behavior. In the elevated plus-maze, the mean time (sec) spent in the open arms (F) and the mean number of open arm entries (G) are measures of anxiety-like responses and were assessed in wild-type and mutant mice. (H) Mean number of entries in the open and closed arms, an indicator of exploratory activity, during the elevated plus-maze task. Data are expressed as mean ± SEM. n = 8-14 (A-E) or 16-17 (F-H) per group.
**Figure 5.7. Deficient social approach behaviors in Srr<sup>Y269*</sup> mutant mice.** Wild-type (+/+) and mutant animals (Y269*/Y269*) mice were assessed in a social affiliations task. *(A)* Mean time (sec) spent in a chamber containing a stranger mouse, a central chamber, and a chamber with an empty cage in the sociability session. *(B)* Mean time (sec) spent in a chamber containing a newly introduced mouse (stranger 2), a central chamber, and a chamber with a familiar mouse (stranger 1) in the social novelty session. Number of chamber entries in the sociability *(C)* and social novelty *(D)* session. *(E)* Mean latency (sec) to uncover a buried food pellet of wild-type and mutant animals during a test of olfactory function. Data are presented as mean ± SEM. n = 13 (A-D) or 14 wild-type and 12 (A-D) or 7 (E) mutant mice; **p < 0.01, ***p < 0.001 compared to the chamber with empty cage or a familiar mouse, within genotype; ##p < 0.01 compared to wild-type mice in the same chamber.
Figure 5.8. Disrupted prepulse inhibition (PPI) of the acoustic startle response in 

$Srr^{Y269*}$ mutant mice. Wild-type (+/+) and mutant animals (Y269*/Y269*) were assessed in a PPI assay using three prepulse intensities (69, 73, 81 dB) (A) Mean % inhibition of the startle response in wild-type and mutant mice. (B) Mean startle amplitude in response to a stimulus (120 dB) given in absence of a prepulse. (C) Mean startle reactivity to a range of stimulus intensities (70 – 120 dB) administered without a prepulse in wild-type and mutant mice. Reactivity to varying stimulus intensities is used to assess hearing capacity. Data are shown as mean ± SEM. n = 9-14 per group; *$p < 0.05$ compared to wild-type mice, within the same prepulse intensity.
Figure 5.9. Mice that lack Srr function have a deficit in spatial object recognition. (A) Mean number of beam breaks, a measurement of locomotion, in an empty open field arena (S1) for wild-type (+/+) and mutant (Y269*/Y269*) mice. (B) Mean duration (sec) of locomotor activity during the sessions of habituation (S2-S4), spatial change (S5), and non-spatial change (S6). (C) Mean duration (sec) of object exploration in the habituation sessions (S2-S4). (D) In the last habituation session (S4) of object recognition task, the mean time (sec) spent exploring the objects that will be displaced or non-displaced in the subsequent spatial change session was evaluated. (E) Mean time (sec) spent exploring the displaced and non-displaced objects in the spatial change session. (F) Mean time (sec) spent exploring the novel item and three familiar objects in the non-spatial change session. Data are expressed as mean ± SEM. n = 8 wild-type and 11 mutant mice; **p < 0.001, ***p < 0.001 compared to the non-displaced objects or the familiar objects, within each genotype; #p < 0.05 compared to wild-type mice exploring the same object category.
Figure 5.10. Impaired spatial memory in *Srr*<sup>Y269*</sup> mutant mice. Wild-type (+/+) and mutant (Y269*/Y269*) mice were assessed in the Morris water maze procedure. (A) Mean path length (m) to reach a target platform in a visible platform session (day 1), and in a hidden-platform acquisition training phase (days 2-8). Spatial memory retention was assessed in the probe trials administered 24 h after days 6 and 8, as indicated by an arrow. Mean % time (sec) spent over a platform area (B) and the number of platform crosses (C) during probe trial 1. Mean % time (sec) spent over a platform area (D) and the frequency of platform crosses (E) during probe trial 2. The dashed line represents chance level, corresponding to the ratio of the target area to the total pool area (2.6%). Data are shown as mean ± SEM. n = 14 wild-type and 13 mutant mice; *p < 0.05 compared to wild-type mice, within the same platform location; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to the target location, within the same genotype.
Pharmacological responses in mutant mice

The efficacy of D-serine (600 mg/kg) to reverse the behavioral impairments in Srr\textsuperscript{Y269*} mutant mice was assessed and compared to clozapine (0.75 mg/kg). Clozapine is a widely used atypical antipsychotic with a pharmacological profile that includes affinity for D\textsubscript{1}- and D\textsubscript{2}-like dopamine receptors and 5-HT\textsubscript{2} receptors\textsuperscript{1}. In the social affiliations task, D-serine fully reversed the social impairment observed in mutant mice (Figure 5.11A). Administration of clozapine to mutant animals, on the other hand, did not significantly induce a preference for the chamber containing the unfamiliar mouse (Figure 5.11A). A significant genotype × apparatus side interaction (F\textsubscript{1,52} = 4.3, p < 0.05) was demonstrated. Wild-type animals given vehicle, D-serine, or clozapine and mutant mice treated with D-serine significantly preferred the chamber with the unfamiliar mouse (p < 0.05), whereas mutant mice injected with vehicle or clozapine did not (Figure 5.11A). The number of chamber entries did not differ significantly between genotype and drug treatment groups, indicating normal exploratory activity (Figure 5.11B). Additionally, pharmacological treatments did not affect olfactory function, as similar latencies to find a buried food pellet were observed (Figure 5.11C).

Deficient PPI in mutant animals was rescued by both D-serine and clozapine administration (Figure 5.12A). There was a significant genotype × drug treatment interaction (F\textsubscript{2,52} = 4.3, p < 0.05), since the PPI deficit in mutant mice was improved by D-serine and clozapine (p < 0.05), to a level comparable to wild-type animals treated with vehicle, D-serine, or clozapine (Figure 5.12A). These compounds did not affect responses to the acoustic startle stimulus (Figure 5.12B).
In the spatial recognition task, impaired reactivity to a spatial change in mutant animals was substantially improved by both D-serine and clozapine. D-serine-treated mutant mice demonstrated reduced locomotor activity in the initial empty open field session ($p < 0.05$; Figure 5.13A); however, no differences in locomotor function were observed in any genotype or drug treatment group during the subsequent object habituation (S2-S4) and spatial change (S5) sessions (Figure 5.13B). In the habituation sessions (S2-S4), the time spent exploring all four objects gradually decreased across sessions similarly (main effect of session: $F_{2,98} = 229.4, p < 0.001$; Figure 5.13C), and no preferential exploration of any individual object was displayed (genotype × drug treatment × session × object interaction: $F_{12,288} = 0.7, p = 0.8$). During the last habituation session (S4), an innate preference for an object category (to be displaced or non-displaced) was not observed in any group (Figure 5.13D). When responses to a spatial change were examined (S5), D-serine and clozapine treatment rescued the spatial recognition deficit in mutant mice (genotype × drug treatment × object category interaction: $F_{2,40} = 4.3, p < 0.05$; Figure 5.13E). Mutant animals treated with D-serine or clozapine spent more time with the displaced than the non-displaced objects ($p < 0.01$), whereas the vehicle-treated mutant animals did not (Figure 5.13E). Recognition of the spatial displacement in D-serine- and clozapine-treated mutant mice was comparable to that of wild-types given vehicle, D-serine or clozapine ($p < 0.05$; Figure 5.13E).

D-serine was also able to reverse the spatial memory deficits of mutant animals in the MWM. In the visible platform session and acquisition training phase, vehicle- and D-serine-treated wild-type and mutant mice had comparable performances (Figure 5.14A). Normal swimming behaviors and search motivation were displayed, as swim speed, floating, and thigmotaxis during these trials was not altered (genotype × drug treatment interaction in
acquisition: swim speed: F_{1,37} = 0.6, p = 0.4, floating time: F_{1,37} = 0.6, p = 0.5, thigmotaxis time: F_{1,37} = 0.01, p = 0.9). After 5 days of acquisition training, the first probe trial was conducted, and D-serine normalized the spatial memory impairment in mutant mice (drug treatment × platform location interaction: F_{1,36} = 4.1, p ≤ 0.05; Figure 5.14B). Compared to vehicle-treated mutant animals, D-serine-injected mutant mice spent more time in a target area 3× the platform diameter (p < 0.05; Figure 5.14B). Furthermore, mutant mice treated with D-serine significantly favored the target location over the averaged analogous non-target areas (p < 0.05), whereas the vehicle-treated mutant mice did not (Figure 5.14B). In the second probe trial performed after 7 days of acquisition training, D-serine again ameliorated spatial memory retention in mutant mice (main effect of drug treatment: F_{1,36} = 5.8, p < 0.05, platform location: F_{1,36} = 13.3, p < 0.001; Figure 5.14C). D-serine-treated mutant animals displayed more time in the target platform area than in the averaged unbiased non-target locations (p < 0.05), while the vehicle-treated mutant animals failed to demonstrate this preference (Figure 5.14C).

The capacity for NMDAR antagonists to induce psychotomimetic effects in humans prompted their application in pharmacological animal models of schizophrenia. In animals, NMDAR antagonists such as MK-801 potentiate a spectrum of behavioral abnormalities relevant to schizophrenia, including locomotor hyperactivity, stereotypy, impaired PPI, and perseverance. To examine the effects of the Srr^{Y269*} mutation in a pharmacological model relevant to schizophrenia, we assessed the effects of MK-801 treatment on PPI and locomotor activity. A low dose of MK-801 (0.1 mg/kg) was chosen to avoid exceeding the range of the behavioral tests measured. Administration of MK-801 was found to produce greater impairments in Srr^{Y269*} mutant mice than in wild-type animals. PPI in mutant mice
given MK-801 was most severely disrupted (Figure 5.15A). There was a significant main
effect of genotype ($F_{1,70} = 18.8, p < 0.001$), drug treatment ($F_{1,70} = 15.6, p < 0.001$), and a
genotype × drug treatment × prepulse intensity interaction ($F_{2,140} = 3.0, p \leq 0.05$). PPI in
mutant mice administered MK-801 was significantly reduced compared to MK-801-treated
wild-type mice and vehicle-treated mutant animals at the prepulse intensities of 69 and 73 dB
($p < 0.05$; Figure 5.15A). Analysis of the acoustic startle response indicated a main effect of
drug treatment ($F_{1,70} = 7.8, p < 0.01$), as startle amplitude in the MK-801-treated mutant mice
was elevated ($p < 0.01$; Figure 5.15B); however, no correlation was found between startle
response and PPI in the mutant mice ($r = -0.06$ for 69 dB, $r = 0.23$ for 73 dB, and $r = 0.09$ for
81 dB; $p > 0.05$). MK-801-induced locomotor hyperactivity was apparent in both wild-type
and mutant mice, but was substantially greater in mutant animals throughout the 30-min
testing period (Figure 5.15C). There was a significant main effect of genotype ($F_{1,33} = 9.5, p
< 0.01$), drug treatment ($F_{1,33} = 35.2, p = 0.001$), and a genotype × drug treatment interaction
($F_{1,33} = 4.0, p \leq 0.05$). Hyperactivity was significantly more prominent in MK-801-treated
mutant mice than in MK-801-treated wild-type mice at the 10 to 30-min time bin ($p < 0.05$;
Figure 5.15C).
Figure 5.11. Social approach behaviors are improved in Srr<sup>Y269*</sup> mutant mice treated with D-serine. Wild-type (+/+) and mutant (Y269*/Y269*) mice given vehicle (veh), D-serine (600 mg/kg; D-s) or clozapine (0.75 mg/kg; cloz) were examined in a test of sociability. (A) Mean time (sec) in a chamber containing a stranger mouse, a central chamber, and a chamber with an empty cage. (B) Number of stranger and empty chamber entries. (C) Mean latency (sec) to dig a buried food pellet in wild-type and mutant mice given vehicle, D-serine, or clozapine in a test of olfactory acuity. Data are presented as mean ± SEM. n = 7-11 per group; *p < 0.05, **p < 0.01, ***p < 0.001 compared to the chamber with empty cage, within genotype and drug treatment group; ##p < 0.01 compared to vehicle-treated wild-type mice in the same chamber.
Figure 5.12. Amelioration of PPI in $Srr^{Y269*}$ mutant mice administered D-serine or clozapine. PPI was measured in wild-type (+/+) and mutant (Y269*/Y269*) mice treated with vehicle (veh), D-serine (600 mg/kg; D-s) or clozapine (0.75 mg/kg; cloz). (A) Mean % reduction in the startle amplitude at prepulse intensities of 69, 73, and 81 dB. (B) Mean startle response to a stimulus (120 dB) given without a preceding prepulse. Data are expressed as mean ± SEM. n = 8-12 per group; **$p < 0.01$, ***$p < 0.001$ compared to vehicle-treated wild-type mice, within the same prepulse intensity; #$p < 0.05$, ##$p < 0.01$ compared to vehicle-treated mutant mice, within the same prepulse intensity.
Figure 5.13. Treatment with D-serine or clozapine normalizes spatial object recognition in $Srr^{Y269*}$ mutant mice. Reactivity to a spatial change was evaluated in wild-type (+/+ ) and mutant (Y269*/Y269*) mice given vehicle (veh), D-serine (600 mg/kg; D-s), or clozapine (0.75 mg/kg; cloz). (A) Mean number of beam breaks, an assessment of locomotion, in an empty open field arena (S1). (B) Mean duration (sec) of locomotor activity in the habituation sessions (S2-S4), and spatial change session (S5). (C) Mean duration (sec) of object exploration in the habituation sessions (S2-S4). (D) In the last habituation session (S4), the mean time (sec) spent exploring the objects that will be displaced or non-displaced in the subsequent session was examined. (E) Mean time (sec) spent investigating displaced and non-displaced objects in the spatial change session (S5). Data are shown as mean ± SEM. n = 8-10 per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the non-displaced objects, within genotype and drug treatment group; ##$p < 0.01$ compared to vehicle-treated wild-type mice exploring the same object category; ~$p < 0.05$ compared to vehicle-treated mutant mice.
Figure 5.14. D-serine treatment improves retention of spatial memory in SrrY269* mutant mice. Wild-type (+/+) and mutant (Y269*/Y269*) mice treated with vehicle (veh) or D-serine (600 mg/kg; D-s) were examined in the MWM task. (A) Mean path length (m) completed to attain a visible (day 1) or hidden platform (acquisition trials, days 2-8) in the training trials. Retention of spatial memory was assessed in probe trials given 24 h after day 6 and 8, as indicated by an arrow. (B) Mean % time (sec) spent in a platform area during probe trial 1. (C) Mean % time (sec) spent in a platform area during probe trial 2. The dashed line marks chance level. Data are presented as mean ± SEM. n = 8-12 per group; *p < 0.05, **p < 0.01 compared to vehicle-treated mutant mice, within the same platform location; #p < 0.05 compared to the target location, within the same genotype and drug treatment group.
**Figure 5.15. Potentiation of behavioral responses relevant to schizophrenia following NMDAR inhibition.** PPI and locomotor activity were measured in wild-type (+/+) and mutant (Y269*/Y269*) animals treated with vehicle or MK-801 (0.1 mg/kg). (A) Mean % inhibition of the acoustic startle response in a PPI assay using prepulse intensities of 69, 73, and 81 dB (n = 16-24 per group). (B) Mean startle amplitude in response to an acoustic stimulus (120 dB) in the absence of a prepulse. (C) Mean number of beam breaks in an empty open field during a 30-min assessment of locomotor activity (5-min bins) (n = 9-10 per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 compared to vehicle-treated wild-type mice, within the same prepulse intensity or time bin; ##p < 0.01, ###p < 0.001 compared to vehicle-treated mutant mice, within the same prepulse intensity or time bin; ~p < 0.05, ~~p < 0.01, ~~~p < 0.001 compared to MK-801-treated wild-type mice, within the same prepulse intensity or time bin.
Discussion

Reduced Srr activity resulted in diminished levels of D-serine and behavioral impairments resembling aspects of the negative and cognitive symptoms of schizophrenia, including social approach deficits, reduced PPI, and deficient spatial recognition and memory. These behavioral deficits could be ameliorated by D-serine and the atypical antipsychotic clozapine, or aggravated by an NMDAR antagonist considered to be a pharmacological model of this disorder. These results point to an important role for Srr in influencing behavioral perturbations associated with schizophrenia.

We identified one functional mutation in the Srr gene in 7502 mice. In ENU mutagenesis, functional mutation rates at a specific locus are typically 1 in 1000 gametes. However, Srr has a small coding region with only 339 codons, and based on the Poisson distribution and our screening of 239 residues within Srr, the probability of discovering only one functional mutation among 7502 mice is 20%261. Consequently, it was not unexpected that we found only one functional mutation in our Srr screen. Though we considered a functional mutation to be one that changes an amino acid in an exon, it has been demonstrated that regulatory elements outside exons can influence gene expression139, and the study of such point mutations may also reveal functional effects. ENU induces random single-base substitutions, and based on the average mutation rate, the F1 founder for the SrrY269* line would have ~29 other functional heterozygous mutations randomly distributed among the ~30,000 genes of the mouse genome356. After the 7 generations of backcrossing onto the C57BL/6J strain, these additional heterozygous mutations would be reduced to 0.23. Moreover, only 25% of the 0.23 additional mutations were expected to be present in both breeding mice of a N8 intercross357, and the use of multiple breeding pairs further ensures
independent segregation of these unlikely mutations. These steps effectively eliminated the likelihood that the abnormal phenotypes found in $Srr^{Y269*}$ mice were related a mutation other than the nonsense mutation in $Srr$. Also, the capacity for D-serine to reverse the behavioral deficits supports that the $Srr$ mutation is responsible for the observed mutant phenotypes.

The degree to which D-serine is depleted in $Srr^{Y269*}$ mice is noteworthy. Both enzyme activity assays and Western blot analysis indicate a complete loss of Srr protein expression, a result that might not be predicted from the introduction of a premature stop signal at codon 269. Mutated mRNAs containing a premature stop codon are detected and degraded by processes collectively termed nonsense-mediated mRNA decay (NMD)$^{358}$. The best-understood mechanism of NMD in mammals relies on proteins that remain at the last splice junction until the first round of translation and thereby detect translational termination occurring greater than 50-55 nucleotides upstream of the last splice junction. Codon 269 is precisely at the beginning of exon 9, the canonical last exon of murine $Srr$, and therefore does not fit this rule. This may signify that there are alternative mechanisms of NMD that depend on somewhat different requirements. It is also possible that a truncated protein is produced from the mutant $Srr$ mRNA but is inherently unstable. Regardless of the process(es) by which Srr protein is depleted in $Srr^{Y269*}$ mice, a detectable level of D-serine remains in the brains of these animals, potentially rendering the phenotype somewhat less severe than NMDA-NR1 ablation. A considerable portion of the residual D-serine may come from the diet, as other D-amino acids can reach the CNS via this route$^{91}$. Alternatively, it has been suggested that the glycine cleavage system or other enzymes may contribute to D-serine levels in the brain$^{359}$. 
In \textit{Srr}^{Y269*} mice, D-serine concentrations were substantially decreased in the hippocampus and frontal cortex, while L-serine levels were elevated in the frontal cortex. Drug-naïve patients with schizophrenia have been found to have similar changes in L- and D-serine\textsuperscript{23, 152}, and although antipsychotics do not directly alter D-serine levels, improvement of schizophrenia symptoms are correlated with an elevation in D-serine\textsuperscript{158}. These changes in D-serine have been proposed to be mediated by alterations in Srr function. In postmortem studies, Srr mRNA and protein levels have been shown to be decreased in the hippocampus of schizophrenia patients\textsuperscript{152}, while some studies report an increase in Srr protein\textsuperscript{94, 153}. Activity of Srr is closely linked to NMDAR activation, as antagonism of the NMDAR has been found to upregulate Srr mRNA and protein expression\textsuperscript{360}. In our study, lack of Srr activity did not produce any detectable changes in the widespread levels of the NMDAR subunit NR1, or in other regulators of NMDAR-mediated neurotransmission, including GluR1, GluR2, GlyT-1, and DAO, although it remains possible that there could be more subtle alterations in specific brain regions or subcellular compartmentalization. In contrast, an increase in PICK1 was observed in \textit{Srr}^{Y269*} animals. In addition to Srr, PICK1 interacts with and regulates numerous other proteins, many of which have been implicated in schizophrenia, including glutamate receptors, dopamine transporters, neuregulin receptors, and ephrins\textsuperscript{143}. The PICK1 gene has been associated with schizophrenia risk\textsuperscript{106} and abnormal PICK1 transcript levels have been reported in the prefrontal cortex of schizophrenia patients\textsuperscript{155}. Thus, the ability of the \textit{Srr} mutation to reproduce some of the changes observed in schizophrenia supports the relevance of this model to the pathophysiology of this disease.

We found that \textit{Srr}^{Y269*} mutant mice displayed behaviors related to the negative and cognitive symptoms of schizophrenia. The social deficits exhibited by mutant animals were
specific to a test of sociability. In the affiliations task, the sociability phase examines the motivation for social encounters, while the social novelty phase assesses social memory and the ability to distinguish a socially novel stimulus. Exploratory activity, olfactory acuity, and anxiety were unaffected in mutant mice, and therefore these parameters are not likely to account for the observed social approach impairment. Mutant animals also displayed a disruption in PPI, a cross-species measure of pre-attentive information-processing. Reductions in PPI are consistently demonstrated in schizophrenia and schizotypal personality disorder, and the degree to which PPI is affected correlates with the severity of symptoms. Since cognitive dysfunctions are recognized to be a primary and enduring core deficit in schizophrenia, animals were evaluated in a visuo-spatial object discrimination task and in the MWM. Performance in the spatial discrimination task has previously been shown to be sensitive to pharmacological manipulation with compounds that affect the dopaminergic and serotonergic system, and is impaired by lesions to areas such as the nucleus accumbens, hippocampus, and prefrontal cortex, that are recognized to be important for cognitive function and implicated in schizophrenia pathogenesis. In this task, mutant mice displayed a selective inability to detect a spatial change. Conversely, the responses of mutant animals to novelty remained unaltered, concordant with their normal ability to detect novelty in a social environment. Additionally, prior studies have shown that NMDAR antagonists are capable of impairing spatial recognition while leaving intact the capacity to respond to nonspatial novelty. These visuo-spatial deficits are further supported by the diminished ability to retain a spatial memory in the MWM. The cognitive deficits displayed in the mutant mice were not related to perturbations in exploration, motivation, or motor coordination, as these animals performed adequately in locomotor
activity, rotarod, and MWM visible platform tests. Furthermore, the deficits in spatial tasks seen in \( Srr^{\gamma 269^*} \) mice may be comparable to the deficits in perception and representation of spatial relationships, motion, and orientation\(^{353, 354} \) that have been described in schizophrenia.

Behavioral impairments in \( Srr^{\gamma 269^*} \) mutant mice were ameliorated by D-serine and clozapine. In the social affiliation task, D-serine was found to be more effective than clozapine at reversing the reduced sociability in mutant animals. The ability of clozapine to normalize social deficits induced by NMDAR antagonists is controversial in rodent studies\(^{333} \), and investigations in patients do not support a direct improvement of primary negative symptoms by clozapine\(^{331} \). In clinical trials with D-serine and other activators of the NMDAR glycine site administered as adjuvant treatments, symptomatic improvements have been observed\(^{11, 179} \). These glutamatergic therapies have demonstrated to be particularly beneficial in alleviating negative and cognitive symptoms, with some studies achieving negative symptom response independent of antipsychotic effects, suggestive of direct improvement in primary negative symptoms\(^{175, 212, 331} \). Likewise, our findings support the efficacy of D-serine to ameliorate these chronically debilitating disturbances.

NMDAR antagonism induced prominent behavioral disturbances in \( Srr^{\gamma 269^*} \) mutant mice, and this may reflect a sensitization of NMDARs in response to decreased stimulation. NMDAR inhibition in humans mimics endogenous symptoms of schizophrenia, and in rodents produces a complex behavioral profile relevant to the disease\(^{10, 227} \). The enhanced effects of MK-801 in mutant mice were most apparent in an assay of locomotor function, where behavioral responses were assessed from a common baseline. Hyperactivity is an animal model for the positive symptoms of schizophrenia that is proposed to correspond to psychomotor agitation\(^{227} \).
In contrast to mice with global losses of NMDA-NR1 subunit\textsuperscript{248}, diminished Srr activity and D-serine do not impact viability nor produce any gross physical abnormalities. Instead, our findings suggest that low D-serine and Srr function is relevant to modulating the more subtle phenotypes characteristic of psychiatric illness. Aberrant regulation of D-serine has also been implicated in neuropathologies involving excitotoxicity, including Alzheimer’s disease, stroke, and amyotrophic lateral sclerosis\textsuperscript{363-365}, and studying the \textit{Srr}\textsuperscript{Y269*} mice could further elucidate the role of altered D-serine in these neurodegenerative diseases. Moreover, \textit{Srr}\textsuperscript{Y269*} mice may be a useful preclinical model to assess the efficacy of novel therapeutic interventions for schizophrenia and other neurological disorders influenced by D-serine availability.
CHAPTER 6

D-serine and genetic inactivation of D-amino acid oxidase enhances reversal learning and extinction in mice

Published as:

Contributions to project

V. Labrie backcrossed, genotyped, and maintained the animal colonies, conducted all behavioral experiments, assisted in the writing of the Neuropsychopharm (Duffy et al. 2008;33:1004-18) manuscript, and wrote the Learn Mem (Labrie et al. 2008;16:28-37) manuscript. S. Duffy performed the electrophysiological experiments, dissection of the brain regions, wrote the Neuropsychopharm manuscript, and helped review the Learn Mem manuscript. G. Rauw performed the HPLC analysis. W. Wang conducted the chemiluminescent assay. E. Weiss assisted with genotyping. R. Konno provided the \( Dao^{1G181R} \) mice on the ddY background. M. Pauley-Evers provided the \( Grin^{1D481N} \) mice. S. Barger, G.B. Baker, and J.C. Roder supervised the experiments conducted in their laboratories.
Summary

Activation of the N-methyl-D-aspartate receptor (NMDAR) glycine site has been shown to accelerate adaptive forms of learning that may benefit psychopathologies involving cognitive and perseverative disturbances. In this study, the effects of increasing the brain levels of the endogenous NMDAR glycine site agonist D-serine, through the genetic inactivation of its catabolic enzyme D-amino acid oxidase (DAO), were examined in behavioral tests of learning and memory. In the Morris water maze task (MWM), mice carrying the hypofunctional $Dao1^{G181R}$ mutation demonstrated normal acquisition of a single platform location, but had substantially improved memory for a new target location in the subsequent reversal phase. Pharmacological treatment with D-serine (600 mg/kg) also specifically improved reversal memory, and rescued deficient MWM acquisition in $Grin1^{D481N}$ mutant mice that have a lower D-serine affinity. Furthermore, $Dao1^{G181R}$ mutant animals exhibited an increased rate of extinction in the MWM that was similarly observed following administration of D-serine (600 mg/kg) in C57BL/6J mice. In contextual and cued fear conditioning, no alterations were found in initial associative memory recall; however, extinction of the contextual fear memory was facilitated in mutant animals. Thus, an augmented level of D-serine resulting from reduced DAO activity promotes adaptive learning in response to changing conditions. The NMDAR glycine site and DAO may be promising therapeutic targets to improve cognitive flexibility and inhibitory learning in psychiatric disorders such as schizophrenia and anxiety syndromes.
Experimental overview

We investigated the effects of diminished DAO function and augmented D-serine in learning and memory in mice. The N-methyl-D-aspartate receptor (NMDAR) has an important role in excitatory neurotransmission and contributes to numerous brain processes, including synaptic plasticity, learning, and memory formation. D-serine is an endogenous co-agonist for the NMDA-NR1 glycine site, acting with high selectivity and a potency similar to or greater than that of glycine. In the brain, the localization of D-serine closely resembles that of NMDARs, and D-serine has been reported to be the predominant physiologic co-agonist for the maintenance of NMDAR-mediated currents in the hippocampus, retina, and hypothalamus. Moreover, in vivo studies have demonstrated that the NMDAR glycine site is not saturated at the synapses of several brain regions. Consequently, increasing D-serine levels may modulate neurotransmission and behavioral responses reliant on NMDAR activity.

The NMDAR glycine site has been implicated in the pathophysiology and treatment of a number of psychiatric conditions. Blockade of the NMDAR with non-competitive antagonists like phencyclidine results in the production and exacerbation of schizophrenic-like symptoms in humans and animals. Genetic studies have associated genes that mediate D-serine synthesis and degradation with a vulnerability to schizophrenia, and levels of D-serine are decreased in the CSF and serum of schizophrenic patients. These observations prompted clinical trials with direct and indirect activators of the NMDAR glycine site, including D-serine, and improvements were revealed when these compounds were added to conventional antipsychotic regimes, particularly with the negative and cognitive symptoms of schizophrenia. Furthermore, altered NMDAR activation has
also been shown to affect extinction, a learning process that may be of benefit in anxiety illnesses, such as post-traumatic-stress syndrome and obsessive-compulsive disorder\textsuperscript{196}. In rodents, extinction was shown to be impaired following inhibition of NMDARs in contextual fear conditioning, inhibitory avoidance, and eyeblink conditioning tasks\textsuperscript{366-368}. In contrast, the partial NMDAR agonist, D-cycloserine, facilitated the extinction of fear memories in rodents and individuals with phobias and other anxiety disorders\textsuperscript{198-200}. Thus, the NMDAR glycine site and its related modulatory proteins may be important targets for the amelioration of psychopathologies involving cognitive dysfunction and maladaptive behaviors.

Endogenous levels of D-serine in the brain are regulated by its catabolic enzyme, D-amino acid oxidase (DAO), by the D-serine synthesis enzyme, serine racemase (Srr), and by neuronal and glial transporters\textsuperscript{79, 369}. Agents targeting such proteins may prove to be an effective method of increasing cerebral D-serine and occupancy of the NMDAR glycine site, that could overcome the difficulties D-serine and similar compounds have with penetrating the blood-brain barrier\textsuperscript{15, 370}. Inhibiting DAO function in the brain is of particularly valuable approach as it would avoid any nephrotoxic effects associated with high levels of systemic D-serine\textsuperscript{371}. DAO is a peroxisomal flavoprotein that at physiological pH is highly selective for D-serine, and in the brain, DAO is located predominantly in astrocytes\textsuperscript{74}. An inverse correlation between the brain distribution of DAO and D-serine evinces the efficacy of this enzyme, with the most abundant DAO expression located in the D-serine-sparse hindbrain and cerebellum\textsuperscript{87, 93}. To study the effects of limiting DAO function, we tested a line of mice carrying a single point mutation (G181R) that results in a complete lack of DAO activity and consequently augmented D-serine in serum and brain\textsuperscript{258, 259}. These mice have previously been shown to exhibit an \textit{in vitro} increase in NMDAR-mediated excitatory postsynaptic currents
in dorsal horn neurons of the spinal cord and an *in vivo* elevation of cGMP that is indicative of augmented NMDAR activity\textsuperscript{119,372}. This demonstrates that reduced DAO function is capable of augmenting NMDAR activation, and it may follow that cognitive and extinction processes influenced by NMDARs are enhanced in *Dao*\textsuperscript{G181R} mutant mice. To investigate this possibility, we assessed the effects of the *Dao*\textsuperscript{G181R} mutation on learning, memory, and extinction in Morris water maze (MWM) and in contextual and cued fear conditioning paradigms.

**Results**

**D-serine measurements in the brains of *Dao*\textsuperscript{G181R} mice**

To determine the effect of the *Dao*\textsuperscript{G181R} mutation on D-serine levels in C57BL/6J mice, HPLC analysis was employed. D-serine concentrations were found to be significantly elevated in *Dao*\textsuperscript{G181R} mutant animals (main effect of genotype: F\textsubscript{1,83} = 43.4, *p* < 0.001) compared to wild-type animals (Figure 6.1). Higher levels were found in whole brain, whole cortex, hippocampus, and especially cerebellum (*p* < 0.05). In contrast, D-serine levels did not differ in the prefrontal cortex and amygdala (*p* > 0.05). No changes were detected when hippocampus was assayed for the concentration of other amino acids, including aspartate, glutamate, L-serine, glutamine, glycine, arginine, alanine, and GABA (*p* > 0.05; Table 6.1). A chemiluminescent assay measuring D-serine catabolism following exposure to the *R. gracilis* DAO enzyme was also used to quantify D-serine in the hippocampus (wild-type: 3.7 ± 0.1 nmol/mg protein; mutant: 4.8 ± 0.2 nmol/mg protein) and whole cortex (wild-type: 2.5 ± 0.2 nmol/mg protein; mutant: 3.8 ± 0.2 nmol/mg protein) of *Dao*\textsuperscript{G181R} mice. D-serine
concentrations were again found to be elevated in mutant animals (main effect of genotype: $F_{1,27} = 65.7, p < 0.001$) in both of these brain regions ($p < 0.01$).

Figure 6.1. D-serine concentrations in the brains of $Dao1^{G181R}$ mice. HPLC was used to measure D-serine levels ($\mu g/g$ tissue) in whole brain and isolated brain regions including whole cortex, prefrontal cortex, hippocampus, amygdala, and cerebellum of wild-type (+/+) and mutant (G181R/G181R) mice. Data are shown as mean ± SEM. $n = 7-9$ per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to wild-type mice, within the same brain structure.
Table 6.1. HPLC analysis of amino acid concentrations in the hippocampus of $Dao^{G181R}$ mice

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>+/+</th>
<th>G181R/G181R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>208.4 ± 10.6</td>
<td>231.3 ± 13.5</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1499.5 ± 127.7</td>
<td>1542.8 ± 121.4</td>
</tr>
<tr>
<td>Glutamine</td>
<td>739.6 ± 107.7</td>
<td>732.3 ± 61.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>62.9 ± 4.3</td>
<td>71.7 ± 6.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.2 ± 0.6</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>64.2 ± 5.2</td>
<td>75.4 ± 5.9</td>
</tr>
<tr>
<td>GABA</td>
<td>224.9 ± 17.7</td>
<td>268.1 ± 27.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean of amino acid concentration (μg/g tissue ± SEM). n = 8 per group.
A loss of DAO function improves spatial reversal memory

We examined the effect of genetic inactivation of DAO and enhanced levels of endogenous D-serine in a hippocampus-dependent MWM behavioral task. Initially, a control experiment was done where animals were placed into the MWM for a 300-sec acclimatization period without the platform. This verified that the Dao1G181R mice did not have an inherent bias for a quadrant location or alterations in swimming behavior (Table 6.2, left column). Additionally, similar performance was observed in a control experiment involving a visible platform session, indicating comparable sensorimotor abilities and motivation (Table 6.2, left column).

Afterwards, spatial acquisition and reversal learning was tested in the MWM on a separate cohort of mice. The Dao1G181R mutation did not affect performance in a visible platform session or during the 7-day acquisition training phase when the platform was hidden, as demonstrated by a similar path length and latency to reach the platform (Figure 6.2A). Also, swim speed, floating time, and thigmotaxis duration were not altered during the acquisition trials, nor in the subsequent reversal training phase (main effect of genotype in acquisition trials: swim speed: $F_{1,24} = 1.2, p = 0.3$, floating time: $F_{1,24} = 0.5, p = 0.5$, thigmotaxis time: $F_{1,24} = 4.1, p = 0.1$; in reversal trials: swim speed: $F_{1,24} = 0.2, p = 0.6$, floating time: $F_{1,24} = 0.1, p = 0.8$, thigmotaxis time: $F_{1,24} = 1.7, p = 0.2$). Spatial memory in the acquisition probe, as measured by the amount of time spent and number of crosses in an area 2× the platform diameter centered over its former location, did not differ between wild-type and mutant animals ($p > 0.05$; Figure 6.2B). Additionally, both genotypes spent more time (main effect of platform location: $F_{1,24} = 44.1, p < 0.001$) and made more crosses over the target platform area (main effect of platform location: $F_{1,24} = 42.7, p < 0.001$) than the
averaged analogous non-target areas \( (p < 0.05); \) wild-type non-target: % time: 3.0 ± 0.4, crosses: 1.9 ± 0.3; mutant non-target: % time: 2.7 ± 0.4, crosses: 1.5 ± 0.2).

A comparable performance between wild-type and mutant animals was also observed during reversal training, where the hidden platform was switched to a new location (Figure 6.2A). However, memory for the new platform location was substantially improved in \( Dao1^{G181R} \) mutant mice compared to wild-type animals in the reversal probe. Mutant mice spent more time (main effect of genotype: \( F_{1,25} = 5.3, p < 0.05 \), genotype × platform location interaction: \( F_{2,50} = 7.9, p < 0.01 \)) and had a greater number of crosses (genotype × platform location interaction: \( F_{2,50} = 4.4, p < 0.05 \)) over the reversal platform area than wild-type animals \( (p < 0.05); \) Figure 6.2C). Wild-type and mutant mice both demonstrated a preference for the reversal target area (main effect of platform location: % time: \( F_{2,50} = 32.3, p < 0.001 \); crosses: \( F_{2,50} = 31.0, p < 0.001 \)) compared to the acquisition target and the averaged unbiased non-target locations \( (p < 0.05) \), indicating that memory for the reversal target location was present in both genotypes, but was particularly increased in mutant animals.
Table 6.2. Control experiments examining performance in acclimatization and visible platform sessions

<table>
<thead>
<tr>
<th>Session</th>
<th>Behavioral variable</th>
<th>Dao1 mice†</th>
<th>C57BL/6J‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+/+</td>
<td>G181R/G181R</td>
</tr>
<tr>
<td>Acclimatization</td>
<td>% Time in area NE¥</td>
<td>0.9 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>% Time in area SE (AT/ ExT)¥§</td>
<td>1.0 ± 0.3</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>% Time in area SW¥</td>
<td>1.5 ± 0.6</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>% Time in area NW (RT)¥§</td>
<td>1.0 ± 0.4</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Path length (m)</td>
<td>47.3 ± 3.6</td>
<td>47.1 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Swim speed (cm/s)</td>
<td>16.0 ± 1.2</td>
<td>15.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Floating (% time)</td>
<td>6.6 ± 5.7</td>
<td>7.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Thigmotaxis (% time)</td>
<td>77.3 ± 6.6</td>
<td>71.0 ± 3.1</td>
</tr>
<tr>
<td>Visible platform</td>
<td>Path length to target (m)</td>
<td>7.8 ± 0.7</td>
<td>8.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Latency to target (s)</td>
<td>43.8 ± 5.5</td>
<td>40.6 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>Swim speed (cm/s)</td>
<td>17.6 ± 1.0</td>
<td>19.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Floating (% time)</td>
<td>9.2 ± 1.8</td>
<td>8.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Thigmotaxis (% time)</td>
<td>43.1 ± 2.6</td>
<td>40.6 ± 4.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. n = 7-10 per group. In the control experiments no differences were found between genotype or drug treatment groups (p > 0.05 for all behavioral variables). In the acclimatization experiment, animals did not preferentially spend more time in any platform area (main effect of platform location: for Dao1^{G181R} mice: F_{3,45} = 2.7, p = 0.1, for C57BL/6J mice: F_{3,36} = 1.8, p = 0.2).

† Control experiments conducted for reversal and extinction studies with Dao1^{G181R} mice (Figure 6.4 and 6.5).
‡ Control experiments conducted for reversal and extinction studies with D-serine treatments (Figure 6.6).
¥ Chance level for each platform location is 2.6% (the ratio of the target area to the total pool area).
§ AT/ExT is the target platform in acquisition and extinction experiments, RT is the target platform in the reversal experiment.
Figure 6.2. *Dao1*<sup>G181R</sup> mutant mice have improved reversal memory in the MWM task.

The path length (A, left panel; m) and latency (A, right panel; sec) to reach a target platform was examined in wild-type (+/+ ) and mutant (G181R/G181R) mice during a visible platform session (day 1), an acquisition training phase (days 2-8) and a reversal training phase (days 10-12). In the acquisition (B) and reversal (C) probe trials, the % time (sec) spent (left panels) and the number of crosses (right panels) over the target area was measured in wild-type and mutant animals. The dashed line represents chance level, corresponding to the ratio of the target area to the total pool area (2.6%). Platform locations include: the reversal target (RT) in the NW quadrant, the acquisition target (AT) in the SE quadrant, and the averaged unbiased non-target areas in the NE and SW quadrants. Data are presented as mean ± SEM. n = 11 wild-type and 15 mutant mice; *p < 0.05, **p < 0.01 compared to wild-type mice within the same platform location; †p < 0.05, ††p < 0.01, †††p < 0.001 —compared to the reversal target within the same genotype.
Spatial reversal learning and memory is enhanced by D-serine administration

Pharmacological treatments of D-serine were used to confirm the effects we observed in the Dao1G181R mice. To this end, we employed our colony of Grin1D481N mice to allow for comparisons with our subsequent experiment that investigates the effects of D-serine on deficient learning. The Grin1D481N mice have been backcrossed to the C57BL6/J strain for 11 generations, making the wild-type Grin1+/+ mice genetically comparable to C57BL6/J mice. In this experiment, spatial reversal learning and memory following D-serine administration was assessed in wild-type Grin1+/+ mice.

At first, a control experiment was conducted confirming that vehicle- and D-serine-treated mice did not express a quadrant bias or abnormalities in swimming behavior (Table 6.2, right column). D-serine treatments also did not affect performance in a visible platform session (Table 6.2, right column), indicating that D-serine does not impair motor coordination, vision, or search motivation.

Afterwards, spatial acquisition and reversal learning were examined in a separate cohort of mice in the MWM task. D-serine treatment (600 mg/kg) did not alter performance in the hidden-platform acquisition training phase, as indicated by the path length (Figure 6.3A, left panel) and latency (Figure 6.3A, right panel) to reach the target platform. Furthermore, D-serine did not affect swim speed or the propensity for the non-searching behaviors of floating and thigmotaxis in the acquisition training phase nor in the reversal training trials (main effect of drug treatment in the acquisition trials: swim speed: $F_{1,40} = 0.3$, $p = 0.6$, floating time: $F_{1,40} = 0.0003$, $p = 1.0$, thigmotaxis time: $F_{1,40} = 0.8$, $p = 0.4$; in the reversal trials: swim speed: $F_{1,21} = 0.01$, $p = 0.9$, floating time: $F_{1,21} = 3.3$, $p = 0.08$, thigmotaxis time: $F_{1,21} = 1.1$, $p = 0.3$). Spatial memory, as measured in the acquisition probe
trial by the time spent within an area 3× the platform diameter centered over the former location, was also not significantly different between vehicle- and D-serine-treated mice ($p > 0.05$; Figure 6.3B). Both experimental groups learned the specific target platform location as evidenced by a significant preference for the target location (main effect of platform location for % time: $F_{1,40} = 16.2, p = 0.001$) compared to the averaged analogous non-target locations ($p < 0.05$; % time of vehicle non-target: $8.3 \pm 0.5$; % time of D-serine non-target: $7.6 \pm 0.5$).

In contrast to acquisition of the first target, D-serine markedly enhanced the rate at which these mice learned the position of a new hidden target in a subsequent reversal learning task (Figure 6.3A) as revealed by both significantly reduced path length and latency on the third day of training (main effect of drug treatment: path length: $F_{1,21} = 6.2, p < 0.05$, latency: $F_{1,21} = 4.9, p < 0.05$). Furthermore, D-serine substantially improved the memory for the new reversal target position (Figure 6.3C). In the reversal probe trial, D-serine-treated mice spent significantly more time (main effect of drug treatment: $F_{1,21} = 5.7, p < 0.05$) in reversal target area than vehicle-treated animals ($p < 0.05$; Figure 6.3C). Both groups spent significantly greater time in reversal platform area (main effect of platform location: vehicle: $F_{1,21} = 18.8, p < 0.001$) compared to the averaged analogous non-target areas ($p < 0.05$). Comparisons between the time spent in the reversal target and the former acquisition target demonstrated that D-serine-treated mice showed a significant preference for reversal target, whereas vehicle-treated mice did not (genotype × platform location interaction: $F_{1,21} = 5.9, p < 0.05$; Figure 6.3C). Animals given D-serine spent more time in the reversal target than in the acquisition target ($p < 0.01$), indicating a specific learning of the reversal platform location.
We next examined the effects of D-serine during a more rigorous reversal task, a delayed-matching-to-place (DMP) version of the MWM, where the location of the hidden platform was changed daily to one of twelve pre-assigned positions (Figure 6.3D). Mice were given 4 trials per day and the path length of each trial was averaged in three blocks (days 1-4, 5-8, 9-12)\(^{373}\). Over the first 4 days of training, the trial to trial path lengths were significantly reduced for both vehicle- and D-serine-treated mice (main effect of trials on day 1-4: F\(_{3,81} = 7.2, p < 0.001\)). The D-serine-injected mice, however, did not reach significance compared to vehicle-injected mice on the non-random trials 2-4 (main effect of drug treatment: F\(_{1,27} = 3.9, p < 0.06\)). Over the next four days (5 to 8), a clear difference in path length was observed, with D-serine-treated mice showing a significantly greater decrease over the non-random trials 2 to 4 (main effect of drug treatment: F\(_{1,27} = 5.2, p < 0.05\), Figure 6.3D1 and 6.3D2). D-serine administration did not significantly affect swim speed (F\(_{1,27} = 2.0, p < 0.2\)), floating time (F\(_{1,27} = 3.8, p < 0.06\)), or thigmotaxis time (F\(_{1,27} = 3.3, p < 0.08\)). With further training (days 9-12), vehicle-treated mice demonstrated similar path lengths to the D-serine-treated animals.
**Figure 6.3. D-serine specifically enhances spatial reversal learning and memory in wild type mice.** Mice were given vehicle or D-serine treatments (600 mg/kg) and tested in MWM tasks. (A) Mean path length (m; left panel) and latency (sec; right panel) to reach a hidden platform (4 trials/day). During this MWM task, the platform was initially in the SE quadrant during the acquisition training trials (days 1-4), and then relocated to the NW quadrant during the reversal training trials (days 6-8). (B) Mean % time (sec) spent in the target platform area during the acquisition probe trial. The dashed line represents chance level, corresponding to the ratio of the target area to the total pool area (6.6%). (C) Mean % time (sec) spent in a platform location during the reversal probe trial. The reversal target (RT) and the acquisition target (AT) are marked. The dashed line indicates the chance level. (D) In a delayed-matching-to-place (DMP) MWM task, the platform was moved daily to a novel location. (D1) Mean path length (m) for each of the four daily trials over days 1-4, 5-8, and 9-12. (D2) Sample swim paths from day 6 trial 2, for a vehicle-treated mouse (top) and a D-serine-treated mouse (bottom). Data are shown as mean ± SEM. n = 23 vehicle- and 19 D-serine-treated mice in the acquisition training and probe, 12 vehicle- and 11 D-serine-treated mice in the reversal training and probe, 16 vehicle- and 13 D-serine-treated mice in the DMP trials; *p < 0.05 vehicle-treated mice compared to D-serine-treated animals; #p <0.05 is the main effect across trials 2-4.
D-serine rescues spatial learning and memory in *Grin1*<sup>D481N</sup> mutant mice

To determine whether our D-serine treatment protocols are capable of modulating spatial acquisition learning and memory, the *Grin1*<sup>D481N</sup> mutant mouse line was used<sup>256</sup>. *Grin1*<sup>D481N</sup> mutant mice have a reduction in D-serine/glycine binding affinity and have previously been demonstrated to have a deficit in spatial acquisition learning<sup>256</sup>. In the MWM, mutant animals demonstrated a significant deficit in acquisition of a hidden platform location that was reversed by D-serine administration by the fourth training day (Figure 6.4A). There was a significant group × days interaction (F<sub>6,180</sub> = 3.8, p < 0.01) and a main effect of group on day 4 (F<sub>2,60</sub> = 4.5, p < 0.05). Vehicle-treated mutant mice had a greater path length reach the target platform on day 4 of acquisition training than vehicle-treated wild-type mice (p < 0.01). In contrast, mutant mice administered D-serine displayed an improved path length on day 4 (p ≤ 0.05) that was comparable to vehicle-treated wild-type mice.

In the subsequent probe trial, *Grin1*<sup>D481N</sup> mutant mice displayed a substantially impaired performance that was ameliorated by D-serine. Vehicle-treated mutant mice spent less time (main effect of group: F<sub>2,59</sub> = 5.2, p < 0.01; Figure 6.4B) and made fewer crosses (vehicle-injected wild type: 6.0 ± 0.5 passes, vehicle-injected mutant: 3.1 ± 0.5 passes, D-serine-injected mutant: 6.4 ± 0.8 passes; main effect of group: F<sub>2,59</sub> = 9.8, p < 0.001) over a target area 3× the acquisition platform diameter than wild-type and D-serine-treated mutant animals (p < 0.05). There were no significant differences in these parameters between vehicle-treated wild-type and D-serine-treated mutant mice, indicating that D-serine rescued the spatial learning and memory deficit in *Grin1*<sup>D481N</sup> mutant animals. These effects are further demonstrated by the spatial probability map derived from the swim paths recorded during the probe trial (Figure 6.4C, lower panel). Additionally, animals given D-serine
demonstrated spatial memory that was specific for the target platform location (main effect of group: $F_{1,59} = 11.4, p < 0.001$). Both vehicle-injected wild-type and D-serine-injected mutant mice showed significant preferences for the target location over the averaged analogous non-target locations ($p < 0.05$), while the mutants given vehicle did not (% time in non-target: vehicle-treated wild-type: $8.3 \pm 0.5$, vehicle-treated mutant: $5.3 \pm 0.4$, D-serine-treated mutant: $8.8 \pm 0.6$).

To verify that the $Grin1^{D481N}$ mutation and D-serine treatment in mutant animals did not affect motor coordination, vision, or motivation, groups were trained for four trials in a visible platform MWM task. The path length to reach the visible target platform did not differ between vehicle-treated wild type, vehicle-treated mutant, and D-serine-treated mutant mice (main effect of group: $F_{2,22} = 0.6, p = 0.6$). In contrast, there was a main effect of trial (pathlength: $F_{3,66} = 10.3, p < 0.001$) indicating that learning in the groups occurred across trials. No differences were found in swim speed ($F_{2,22} = 2.9, p = 0.07$), floating time ($F_{2,22} = 1.1, p = 0.3$), and thigmotaxis time ($F_{2,22} = 1.6, p = 0.2$) between any group.

In sum, these results indicate that exogenous D-serine can rescue spatial acquisition learning, but only when endogenous D-serine is limiting, in this case by a reduced NR1 affinity.
Figure 6.4. D-serine treatment rescues deficient spatial learning and memory in *Grin1*<sup>D481N</sup> mutant mice. Performance in the MWM task was examined in vehicle-treated wild-type mice and mutant mice given vehicle (veh) or D-serine (600 mg/kg; D-s) treatments. (A) Mean pathlength (m ± SEM) during the hidden platform search (day 1-4). (B) Mean % time (sec ± SEM) spent in the target platform area during the probe trial administered 24 h after day 4. The dashed line is the chance level (6.6%). (C) Swim paths in the probe trial. Upper panels: typical swim paths during the probe trial for a vehicle-treated wild-type mouse (left), a mutant mouse treated with vehicle (middle), and a mutant mouse treated with D-serine (right). The gray disk in the SE quadrant demarcates the target platform area (3 × platform diameter) centered over the former platform location. Lower panels: the spatial probability map for all animals in each group during the probe trial. Each map has a spatial resolution of approximately 5.8 cm (each pixel maps a pool area of 5.8 × 5.8 cm). The green color includes the chance probability level. The highest probability (orange-red) for vehicle-treated wild-type mice and D-serine-treated mutant animals lies over the former platform location (gray disc, upper panels). The equivalent area for the vehicle-treated mutant mice is at chance level (green). A second area of high probability overlies the release point (NW periphery) in all three maps. n = 23 vehicle-treated wild-type, 24 vehicle-treated mutant, and 16 D-serine-treated mutant animals; *p < 0.05, **p <0.01 mutant mice injected with vehicle compared to vehicle-injected wild-type mice; #p <0.05, ##p <0.01 mutant mice injected with vehicle compared to D-serine-injected mutant mice.
Extinction learning is facilitated in mice that lack DAO activity

Considering that the *Dao1*<sup>G181R</sup> mutant mice expressed a specific improvement in reversal memory and the recent literature describing an enhancing effect of exogenous D-cycloserine on the extinction of learned responses<sup>196</sup>, the *Dao1*<sup>G181R</sup> mice were examined in an extinction task in the MWM using repeated probe trials. The extinction procedure employed an experimentally-naïve cohort of mice and was a separate experiment from the reversal learning study. In the visible and acquisition phases, wild-type and mutant mice demonstrated a similar performance, as measured by the path length and latency to find the visible or hidden platform (Figure 6.5A). In the first probe trial conducted 24 h after the completion of acquisition training, wild-type and mutant mice did not differ in the time spent or number of crosses over a target area 2× the platform diameter, signifying that the memory for the target platform was initially equivalent in both genotypes (*p* > 0.05; Figure 6.5B, 6.5C). In this probe trial, wild-type and mutant mice also displayed a greater amount of time (main effect of platform location: *F*<sub>1,14</sub> = 24.7, *p* < 0.001) and crosses in the target location (main effect of platform location: *F*<sub>1,14</sub> = 23.4, *p* < 0.001) than in the averaged analogous non-target areas (*p* < 0.05; wild-type non-target: % time: 3.0 ± 0.6, crosses: 1.8 ± 0.3; mutant non-target: % time: 2.4 ± 0.4, crosses: 1.6 ± 0.2). During the extinction probe trials, *Dao1*<sup>G181R</sup> mutant mice demonstrated enhanced extinction rates compared to the wild-type animals. The mutant animals spent less time (main effect of genotype: *F*<sub>1,14</sub> = 3.7, *p* < 0.05) and made fewer crosses over the target location (main effect of genotype: *F*<sub>1,14</sub> = 5.3, *p* < 0.05), particularly on the fourth and fifth probe repeat (days 7 and 9, *p* < 0.05; Figure 6.5B, 6.5C). Though extinction of the learned response occurred earlier in the mutant mice, it was demonstrated across trials in both genotypes (wild-type: main effect of trial: % time: *F*<sub>7,49</sub> =
2.7, $p < 0.05$, crosses: $F_{7,49} = 5.2, p < 0.001$; mutant: main effect of trial: % time: $F_{7,49} = 4.6, p < 0.001$, crosses: $F_{7,49} = 6.4, p < 0.001$). Swim speed, floating, and thigmotaxis time did not differ between genotypes during the extinction phase (main effect of genotype: swim speed: $F_{1,14} = 0.8, p = 0.4$, floating time: $F_{1,14} = 0.2, p = 0.7$, thigmotaxis time: $F_{1,14} = 0.2, p = 0.7$).

Additionally, comparisons were made between mutant animals that received several extinction sessions and control mutant animals given a single probe trial on day 9. A greater time ($p < 0.05$) and number of crosses in the target area ($p < 0.01$) was observed in the control mutant mice, indicating that $Dao1^{G181R}$ mutant animals exhibit a facilitation in extinction rather than an impairment in memory duration.
Figure 6.5. The $Dao^{G181R}$ mutation augments extinction responses in the MWM. The path length (A, left panel; m) and latency (A, right panel; sec) to attain a target platform was evaluated in wild-type (+/+ and mutant (G181R/G181R) mice during the visible platform session (day 1), and acquisition training phase (days 2-8). Multiple probe trials were performed on alternate days (1-15), allowing extinction to be examined in the $Dao^{G181R}$ wild-type and mutant mice. In a control group of mutant mice, a probe trial was given only on day 9. During the probe trials, the time (sec) spent (B) and frequency of crosses (C) over the target area was determined. Chance levels are depicted as a dashed line. Data are expressed as mean ± SEM. n = 8 wild-type, 8 mutant, and 6 mutant control mice; *$p < 0.05$, **$p < 0.01$ compared to wild-type mice within the same day; #$p < 0.05$, ##$p < 0.01$ compared to mutant mice that received several extinction trials.
D-serine treatments enhance extinction learning

To confirm that a loss of DAO activity produces an increase in the extinction of a learned response, D-serine treatments were given during the probe sessions to wild-type C57BL/6J mice. In the first probe trial following acquisition training, a similar amount of time and number of crosses over an area 2× the platform diameter was observed in vehicle- and D-serine-injected mice, suggesting that the initial memory for the platform location was comparable in each group (p > 0.05; Figure 6.6A and 6.6B). Accordingly, both treatment groups spent more time (main effect of platform location: F1,15 = 22.6, p < 0.001) and made more crosses over the target area (main effect of platform location: F1,15 = 33.3, p < 0.001) than in the averaged analogous non-target locations during the first probe trial (p < 0.05; vehicle non-target: % time: 3.7 ± 0.7, crosses: 1.7 ± 0.3; D-serine non-target: % time: 3.2 ± 0.5, crosses: 2.0 ± 0.3). In the extinction probe sessions, D-serine-treated animals displayed an enhancement in extinction, as measured by the reduced time spent (main effect of genotype: F1,15 = 6.8, p < 0.05) and lower frequency of crosses over the target platform area (main effect of genotype: F1,15 = 7.9, p < 0.05) (Figure 6.6A and 6.6B). Compared to vehicle-treated mice, animals given D-serine had a reduction in the amount of time spent in the target location on days 3, 5, 9, and 15 (p < 0.05), and crossed the platform area less often on days 9 and 15 (p < 0.05). Though greater in the D-serine-injected mice, extinction was progressively demonstrated across probe sessions in both treatment groups (vehicle: main effect of trial: % time: F7,56 = 4.3, p < 0.001, crosses: F7,56 = 5.7, p < 0.001; D-serine: main effect of trial: % time: F7,49 = 6.7, p < 0.001, crosses: F7,49 = 7.1, p < 0.001). Additionally, D-serine-treated animals that received a single extinction session on day 9 had a greater % time (p < 0.01) and number of crosses over the target location (p < 0.001) compared to mice that received
multiple extinction sessions. This indicates that the effects of D-serine were specifically related to extinction rather than forgetting over the passage of time.

![Graphs showing the effects of D-serine treatment on extinction rates in the MWM.](image)

**Figure 6.6. D-serine treatment enhances extinction rates in the MWM.** Male C57BL/6J mice were treated with vehicle or D-serine (600 mg/kg) before each probe trial (alternate days 1-15) and extinction was evaluated in these mice. Also, a control group of mice was administered D-serine injections in the home cage (alternate days 1-7) and was given a probe trial only on day 9. The amount of time (sec) spent (A) and number of crosses (B) over the target area was determined for each treatment group. The dashed line indicates chance level. Data are shown as mean ± SEM. n = 9 vehicle, 8 D-serine, and 7 D-serine control mice; *p < 0.05, **p < 0.01 compared to vehicle-treated mice within the same day; ##p < 0.01, ###p < 0.001 compared to D-serine-treated mice that received several extinction trials.
Absence of DAO activity augments extinction of contextual fear memory

Dao1G181R mice were examined in a fear conditioning paradigm to determine whether our results could extend to another hippocampus-dependent task with different sensory, motivational, and performance requirements. In fear conditioning experiments, the hippocampus is known to be required for the formation and retrieval of context-fear associations, while the amygdala is necessary for conditioning and recall of contextual and cued/tone associations. An assessment of freezing time in the training phase revealed that the Dao1G181R mutation did not produce any non-specific effects on fear conditioning, as no differences in freezing time were observed during the 2-min baseline activity period, the 30-sec auditory tone exposure, or the 30-sec interval following shock presentation ($p > 0.05$; Figure 6.7A). In subsequent sessions, context- and tone-shock associations were evaluated, in which contextual freezing responses were normalized to the baseline activity period, while tone freezing responses were relative to the pre-tone period and assessed in an altered context. Wild-type and mutant mice reexposed 24 h later to the training context did not demonstrate differing freezing responses, indicating a similar learning of the context-shock association ($p > 0.05$; Figure 6.7B). However, when the animals were exposed to the context in repeated extinction trials, the Dao1G181R mutant mice demonstrated an enhanced extinction rate (main effect of genotype: $F_{1,29} = 4.6, p < 0.05$) that was especially significant on the second exposure (day 3, $p < 0.05$; Figure 6.7B). Contextual extinction across trials was present earlier in mutant animals (main effect of trial: $F_{4,64} = 3.9, p < 0.01$), but also eventually occurred in wild-type animals (main effect of trial: $F_{4,52} = 6.6, p < 0.001$). Supporting the interpretation of facilitated fear extinction, the control mutant group exposed to the context only on day 7 showed a greater freezing time than mice given several
extinction trials \((p < 0.05)\). In contrast, the tone-shock association and its extinction were not significantly altered in \(Dao1^{G181R}\) mutant mice compared to wild-type littermates \((p > 0.05;\) Figure 6.7C). Since altered pain sensitivity can potentially influence performance in fear conditioning tests\(^{274}\), the tail-flick assay was used to examine antinociceptive responses in the \(Dao1^{G181R}\) mice. Latencies following immersion in hot water were similar in wild-type and mutant mice, suggesting an equivalent pain sensitivity in both genotypes (wild-type: \(14.6 \pm 1.6\) sec, \(n = 17\); mutant: \(13.2 \pm 1.7\) sec, \(n = 15\); \(p > 0.05\)).
Figure 6.7. Loss of DAO activity facilitates the extinction of contextual fear memory.

Freezing responses in wild-type (+/+) and *Dao1*\(^{G181R}\) mutant (G181R/G181R) animals were measured during a training session (A) that contained a baseline activity period, followed by a 30-sec exposure to an auditory tone that co-terminated with a foot-shock (US). The % time (sec) spent freezing to the context (B) and to the tone in an altered context (C) was evaluated in wild-type and mutant mice during the extinction trials (days 1, 3, 5, 7, and 13). Freezing responses were also measured in a control group of mutants exposed to the context and tone on day 7 only. Data are presented as mean ± SEM. n = 14 wild-type, 17 mutant, and 17 mutant control mice; *p < 0.05 compared to wild-type mice within the same day; #p < 0.05 compared to mutant mice that received multiple extinction trials.
Discussion

Diminished DAO function was associated with elevated levels of D-serine in the brain and enhancements in reversal memory and extinction learning in the MWM and contextual fear conditioning procedures. Moreover, pharmacological administration of D-serine improved reversal memory and substantially accelerated extinction of a learned spatial response in the MWM. These findings indicate that D-serine and decreased DAO activity improves performance under changing conditions, and predict their therapeutic utility for the treatment of psychiatric disorders characterized by cognitive inflexibility and aberrant repetitive behaviors.

DAO inhibition may be a well-tolerated and effective means of modulating D-serine levels and NMDAR glycine site occupancy for clinical applications\textsuperscript{15, 196, 371}. Animals that chronically lack DAO activity exhibit normal development, longevity, and reproductive potential\textsuperscript{257}. Furthermore, chronic administration of D-serine at therapeutic doses has not been shown to produce adverse effects in humans\textsuperscript{178}. However, under conditions that promote excitotoxicity and neuroinflammation, D-serine can potentially compromise neuronal survival through excessive NMDAR activation\textsuperscript{364, 369}, suggesting that modest rises in D-serine, as observed following DAO reduction, may be advantageous over dramatic increases. Though D-serine elevations in $Dao^{G181R}$ mutant mice were shown to be greatest in the cerebellum, as expected based on DAO expression patterns\textsuperscript{93}, increases were also demonstrated in the hippocampus, an area crucial to the behavioral tasks studied\textsuperscript{374, 375}. Previous reports have indicated more subtle D-serine increases in the forebrain of $Dao^{G181R}$ mutant mice\textsuperscript{359}, but this difference may be related to background mouse strain. Importantly, reduced DAO activity in mutant mice produced increases in D-serine that enhanced NMDAR
function\textsuperscript{119,372} without producing functional changes in $[^3]$H-D-serine reuptake or alterations in the expression of proteins relevant to the NMDAR signaling, including NR1, serine racemase, glycine transporter 1, and alanine-serine-cysteine transporter 1\textsuperscript{372}. This suggests a lack of obvious compensatory changes in the $\text{Dao1}^{G181R}$ mice. Hence, these animals may be a useful preclinical model for the study of behavioral endophenotypes specifically related to diminished DAO function.

This study is the first to demonstrate that inactivation of DAO leads to improved behavioral flexibility in response to changing environmental contingencies. In the reversal phase of the MWM, memory for the novel platform location was selectively enhanced in $\text{Dao1}^{G181R}$ mutant mice. Acute administration of D-serine was also shown to enhance reversal learning in the MWM in wild-type mice. Prior studies investigating genetically modified and wild-type mice have attributed performance in the reversal MWM task to the capacity for cognitive flexibility, i.e. the ability to simultaneously inhibit a previously acquired spatial navigation strategy and develop a new strategy\textsuperscript{279,376}. Deficits in reversal learning and perseveration have been reported in both rodents and humans given NMDAR antagonists\textsuperscript{231,243,377}, as well as in rats with lesions to the fimbria-fornix or hippocampus\textsuperscript{375}. Exogenous D-serine administration in rats treated with the NMDAR antagonist phencyclidine has been shown to improve impaired reversal learning in the MWM\textsuperscript{243}. Additionally, nitric oxide (NO) has been shown to promote DAO function and suppress serine racemase activity, and antagonism of NO synthase was found to rescue phencyclidine-induced impairments in MWM reversal learning\textsuperscript{378,379}. By directly targeting DAO function, our study indicates that chronically enhanced D-serine levels can also promote appropriate reversal behaviors and suggests that DAO inhibition may attenuate symptoms of cognitive
inflexibility, such as those commonly observed in patients with schizophrenia\textsuperscript{380}. Indeed, a clinical study demonstrated that D-serine administration improved the performance of schizophrenic individuals in the Wisconsin card sorting task, a widely used measure of flexibility\textsuperscript{178}.

Extinction is regarded as a distinct form of learning that acts to suppress, but not erase, previously learned responses\textsuperscript{196}. Like other forms of learning, extinction has been shown to depend on glutamate NMDAR-associated signaling pathways, gene expression, and protein synthesis\textsuperscript{196}. A growing body of evidence indicates that the NMDAR glycine site may be particularly important in mediating the extinction of fear memory\textsuperscript{196, 199}. The partial NMDAR agonist D-cycloserine has been shown to facilitate fear extinction and exposure therapy, though it is not yet clear whether the improvements are related to an increase of NMDAR function during the extinction process\textsuperscript{198-200}. Here, we show that elevated D-serine can accelerate extinction in spatial and fear-based tasks. The improvements were demonstrated to be specifically related to an increase in extinction learning rather than to a deficit in memory duration. Furthermore, the ability of acute D-serine treatments to replicate these findings in wild-type mice indicates this behavioral phenotype is not likely the result of potential abnormalities due to increased D-serine levels during development. Also, facilitated extinction in Dao1\textsuperscript{G181R} mutant mice could not be attributed to changes in motivation or anxiety, as no differences were observed in the incentive to find a visible platform, or in behaviors indicative of anxiety, such as thigmotaxis and baseline freezing. Although we did not detect differing responses in an assessment of antinociception, mutant mice have previously been reported to have elevations in pain sensitivity\textsuperscript{119}, which might bias towards the persistence of a fear response.
Genetic perturbation of DAO activity specifically improved reversal memory and extinction without altering performance during the acquisition trials. Activation of the NMDAR glycine site has been shown to be necessary for the acquisition and memory retrieval of a single platform location. In support, mice with a reduction in NR1 glycine affinity had profound deficits in acquisition learning and memory. However, the ability of glycine site activators to potentiate this type of spatial learning and memory beyond normal capacity remains controversial, and discrepancies may reflect different procedural demands, dosing regimes, or species. In our experiments, D-serine was capable of augmenting acquisition learning only under conditions of limiting D-serine, as in the Grin1 mice, but did not further ameliorate normal levels of acquisition learning. However, in the acquisition phase of the Dao1 studies, animals were trained to achieve asymptotic performance in order to assess reversal learning and extinction from a common baseline. Consequently, we cannot preclude a possible enhancement of normal acquisition by D-serine under some conditions, and others have shown that elevated D-serine can enhance acquisition of spatial learning. Regardless, we demonstrate that elevations in D-serine are not required during the acquisition sessions in order to improve extinction learning, further supporting a vital role in adaptive behaviors necessary for changing contingencies.

Activation of NMDARs is critically involved in the synaptoplastic processes underlying the acquisition of novel behaviors. Recent studies suggest that behavioral alterations in response to changing environmental conditions are also dependent on NMDAR activity, though the neurocellular mechanisms are unclear. Activation of preexisting circuits encoding memory traces renders them subject to modification, a process termed reconsolidation. NMDAR antagonists, including compounds that target NR2B receptors
prevent the induction of memory lability\textsuperscript{387}. Consequently, increased D-serine may augment the propensity for NMDAR-dependent reconsolidation. NMDAR-mediated long-term depression (LTD) is a one form of synaptic plasticity that may contribute to behavioral flexibility by facilitating the remodeling of labile circuits. Alternatively, LTD may facilitate learning under changing environmental conditions by suppressing the expression of previously encoded memory traces. In either case there is compelling evidence for the role of LTD in behavioral flexibility and inhibition. Transgenic mice that exhibit deficits in hippocampal LTD demonstrated a parallel disruption in the ability to learn novel platform locations in the MWM\textsuperscript{385, 388, 389} and a deficiency in acquisition and recall of fear extinction\textsuperscript{390}. Blockade of NMDA-NR2B receptors has been reported to inhibit hippocampal LTD in the adult mouse and has also been found to disrupt behavioral inhibition in a serial reaction task, spatial reversal learning, and fear extinction learning and retention\textsuperscript{279, 337, 338}. Conversely, mice overexpressing NR2B receptors exhibit better learning and memory in tasks that include extinction of fear associations\textsuperscript{255}. Likewise, exogenous application of D-serine was shown to strongly enhance NR2B-dependent LTD in rodent hippocampal slices \textit{in vitro}\textsuperscript{279, 384}. Finally, the propensity for LTD has been shown to increase with exposure to a novel environments and when salient environmental cues are relocated\textsuperscript{391, 392}.

In conclusion, elevated D-serine and genetic inactivation of DAO function ameliorated cognitive flexibility and inhibitory learning in the MWM and in a contextual fear conditioning task. Growing evidence indicates that reduced occupancy of the NMDAR glycine binding site may be involved in the pathophysiology of schizophrenia, and activators that target this site may have therapeutic potential\textsuperscript{12, 15}. Furthermore, modulation of the NMDAR glycine site has also been proposed to benefit extinction and exposure therapy,
which are used to treat several psychopathologies, including obsessive-compulsive disorder and post-traumatic stress syndrome\textsuperscript{199, 200}. The improvement of adaptive responses through the inhibition of DAO activity may be of substantial clinical utility for the treatment of these and other psychiatric illnesses involving an inability to attenuate persistent maladaptive behaviors.
CHAPTER 7

General discussion

Published as:

These studies examined the behavioral effects of pharmacological and genetic manipulation of the NMDAR glycine site in mice. Reduced glycine site affinity via the \textit{Grin1}^{D481N} mutation or diminished D-serine synthesis via the \textit{Srr}^{Y269*} mutation led to behavioral phenotypes reminiscent of some of the negative and cognitive symptoms of schizophrenia. Administration of the direct glycine site agonist D-serine or the GlyT-1 inhibitor ALX-5407 rescued the abnormal phenotypes in genetic animal models with reduced NMDAR function and in mice treated with the noncompetitive NMDAR antagonist MK-801. Furthermore, inactivation of DAO increased brain levels of D-serine and improved adaptive forms of learning that are pertinent to schizophrenia and anxiety disorders. Similarly, exogenous D-serine treatments in wild-type mice enhanced learning under changing conditions. Together, these findings indicate that lowered occupancy of the NMDAR glycine site and decreased D-serine availability may contribute to the pathophysiology of certain clinical features of schizophrenia. Activation of the glycine site by D-serine or DAO inhibition may be an effective therapeutic approach to ameliorate the negative and cognitive symptoms of schizophrenia, and may benefit other psychiatric disorders characterized by cognitive inflexibility and persistent maladaptive behaviors.

**Animal models relevant to the NMDAR glycine site**

Several genetic animal models examining the effects of aberrant function of the NMDAR glycine site and its modulatory enzymes have been developed (Table 7.1). \textit{Grin1}^{D481N} mutant mice with a five-fold reduction in NMDAR glycine affinity display behavioral abnormalities that include persistent latent inhibition (LI) and impairments in sociability, spatial recognition, learning, and memory\textsuperscript{279,280}. LI persistence is also seen in
mice administered the highly selective NMDAR glycine site antagonist L-701,324\textsuperscript{280} and the NMDAR channel blocker MK-801\textsuperscript{281}. Prolonged LI has been associated with deficits in information-processing and cognitive flexibility observed in schizophrenia patients\textsuperscript{297,298}, and has been correlated with the severity of negative symptoms\textsuperscript{299,304}. Furthermore, sociability deficits in animals, as seen in the Grin\textsubscript{1D481N} mice, resembles the social withdrawal aspect of the negative symptoms of schizophrenia\textsuperscript{333}. Impaired sociability is also seen in mice carrying an N-nitroso-N-ethylurea (ENU)-induced point mutation in the Srr gene that results in a loss of Srr activity and lowered D-serine levels\textsuperscript{140}. Furthermore, the Srr\textsuperscript{Y269*} mice demonstrate a deficit in sensorimotor gating, a reflexive and pre-attentive form of information-processing reliant on brainstem circuits\textsuperscript{393} that is commonly disrupted in patients with schizophrenia\textsuperscript{361}. Like the Grin\textsubscript{1D481N} mice, animals with the Srr\textsuperscript{Y269*} mutation display dysfunctions in cognitive ability that include deficits in spatial object discrimination, and long-term memory\textsuperscript{140,394}. Thus, these genetic models of reduced NMDAR glycine site occupancy demonstrate disturbances related to schizophrenia, particularly to the negative and cognitive symptoms, suggesting that the glycine site and its modulators may be relevant to the pathophysiology of these symptoms. An important limitation of these models is the apparent lack of behavioral phenotypes recapitulating the psychotic features of schizophrenia. Mice with the Grin\textsubscript{1D481N} and Srr\textsuperscript{Y269*} mutation do not display an increase in locomotor activity, a behavioral measure relevant to psychomotor agitation\textsuperscript{227} and do not exhibit a loss of LI that is observed in patients with prevalent psychotic symptoms and in rodents administered amphetamine\textsuperscript{297-299}. Consequently, further investigation is required to determine the effect of aberrant glycine site function in these models on other neurotransmitter systems implicated in schizophrenia. In contrast, Grin\textsubscript{1D481N/K483Q} mice that
have two point mutations in the NMDAR glycine site and more severe reductions in glycine site occupancy, demonstrate abnormalities related to psychosis, including locomotor hyperactivity, enhanced stereotypy, and striatal dopaminergic and serotonergic hyperfunction\textsuperscript{340}. However, these animals also demonstrate a resistance to antipsychotics and behavioral deficits that are so profound\textsuperscript{340}, that their relevance to schizophrenia symptomatology is limited.

Animal models with enhanced activation of the NMDAR glycine site demonstrate a resistance to schizophrenia-like symptoms and improvements in cognitive performance. 

$Dao1^{G181R}$ mice lack functional DAO, resulting in an elevation in brain D-serine levels\textsuperscript{259, 395} and an \textit{in vivo} augmentation of NMDAR function\textsuperscript{372}. These mice have been reported to be resistant to the psychotomimetic effects of MK-801\textsuperscript{372, 396} and methamphetamine\textsuperscript{397}, as indicated by an attenuation of drug-induced hyperactivity and stereotypy. Likewise, pharmacological administration of D-serine has been shown to reverse behavioral deficits induced by MK-801 or genetic diminution of NMDAR glycine site activation\textsuperscript{280, 281}. For example, D-serine normalized MK-801-induced hypermotility, persistent LI, and novelty discrimination deficits in wild-type mice\textsuperscript{228, 281, 398}, and reversed the impairments in sociability, sensorimotor gating, spatial recognition and memory in $Grin1^{D481N}$ and $SrrY^{269*}$ mutant mice\textsuperscript{140, 280}. Furthermore, $Dao1^{G181R}$ mutant animals display enhancements in spatial reversal learning and increased extinction rates that are similarly observed in C57BL/6J mice given exogenous treatments of D-serine\textsuperscript{279, 395}. Consequently, DAO inactivation and augmented D-serine concentrations may normalize NMDAR hypofunction and cognitive inflexibility in schizophrenia. Moreover, the improvements in reversal learning and extinction imply that DAO inhibition and D-serine may be useful for the treatment of other
psychopathologies involving persistent maladaptive behaviors, such as obsessive-compulsive disorder and post-traumatic stress syndrome. Though D-serine and similar compounds have demonstrated promising effects in clinical trials with medicated schizophrenia patients\textsuperscript{11, 178, 179}, there are issues regarding the administration of these compounds, as large doses are required in order to penetrate the blood-brain-barrier. In contrast, DAO inhibitors cross the blood-brain-barrier readily\textsuperscript{399, 400}, but whether DAO inhibitors will sufficiently increase levels of D-serine to alleviate disease symptoms in patients has yet to be examined.

Recently, transgenic mice carrying the human $G72$ genomic region have been developed\textsuperscript{401}. In the brains of these mice, $G72$ transcripts were most abundant in the cerebellum, hippocampus, cortex, and olfactory bulb, while in the periphery $G72$ was elevated in the heart and spleen\textsuperscript{401}. Several phenotypes relevant to psychiatric disease were displayed in the $G72$-expressing mice, including sensorimotor gating disruption, enhanced sensitivity to PCP, and an increase in compulsive behaviors\textsuperscript{401}. Further analysis of these mice may uncover the molecular and cellular functions of $G72$ in vivo, and clarify the mechanism by which $G72$ induces behavioral disturbances in the mouse.

Homozygous deletion of all GlyT-1 subtypes in mice results in severe motor and respiratory deficits leading to death on the first postnatal day\textsuperscript{402}. However, heterozygote GlyT-1 knock-out mice are fully viable\textsuperscript{403}, as are mice with a selective elimination of GlyT-1 in the forebrain\textsuperscript{404}. Reduced GlyT-1 expression led to a corresponding increase in glycine availability and augmented NMDAR-evoked excitatory postsynaptic currents in hippocampal slices\textsuperscript{403, 404}. Lower levels of GlyT-1 also enhanced cognitive performance in several behavioral tasks\textsuperscript{403-405}. Heterozygote GlyT-1 knock-out mice demonstrated greater spatial memory retention and a decreased sensitivity to amphetamine-induced disruption of
sensorimotor gating\textsuperscript{403}. Additionally, mice with a loss of forebrain GlyT-1 showed improvements in LI, spatial and object recognition memory, and a resistance to PCP-induced hyperactivity\textsuperscript{404, 405}. The procognitive effects of diminished GlyT-1 activity have also been reported in pharmacological studies. Treatment with GlyT-1 antagonists potentiates LI in rodents under parametric conditions that reduce LI in controls, and reverses LI abnormalities, spatial memory deficits, and hyperlocomotion induced by MK-801 or amphetamine\textsuperscript{281, 406-409}. In neurodevelopmental models of schizophrenia, inhibition of GlyT-1 normalizes impairments in sensorimotor gating, LI, and social recognition memory in adult rats\textsuperscript{311, 406, 408}. However, usage of GlyT-1 inhibitors in clinical settings may merit caution, as animal studies have found that high doses of a GlyT-1 antagonist, such as ALX-5407 or LY2365109, induces compulsive over-activity (akathisia)\textsuperscript{281, 409}, which may be related to spill-over onto inhibitory glycine A receptors\textsuperscript{410}. Rodents given a high dose (10 mg/kg) of ALX-5407 were demonstrated to have high glycine levels in caudal brain regions along with respiratory and motor impairments\textsuperscript{410}, suggesting an increased modulation of glycine A receptors.
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Abbreviations: NMDAR, N-methyl-D-aspartate receptor; KO, knockout; PPI, prepulse inhibition; LI, latent inhibition. Not listed are several genetic models demonstrating that aberrant NR1 function affects behaviors relevant to drug addiction and nociception.
Comparison of animal models examining genes related to the NMDAR glycine site and other genetic models based on schizophrenia susceptibility genes

Since schizophrenia is a complex disorder involving both genetic and environmental factors, manipulation of one genetic or neurochemical pathway is unlikely to replicate all aspects of the disease. Instead, targeting specific pathways may reveal their importance in specific clinical features associated with schizophrenia. Overall, marked reduction in the function of the NMDAR glycine site and in the activity of D-serine modulatory enzymes appear to recapitulate some of the neurocognitive abnormalities and negative symptoms characteristic of schizophrenia. Deficits in spatial learning and memory as well as social interactions are particularly apparent, although this may reflect the choice in assessed behavior and the limitations in valid behavioral paradigms. Models affecting NMDAR glycine site activity also demonstrate an altered sensitivity to psychotomimetic agents and, in general, are responsive to antipsychotic treatment. However, consistent with being a genetically heterogeneous disease, many other candidate susceptibility genes have been implicated in the pathophysiology of schizophrenia\(^1,2\), suggesting the involvement of a synergistic interaction of several risk genes and neurotransmitter systems.

Discoveries in human genetic studies have fueled the development of several genetic mouse models based on schizophrenia susceptibility genes. Among the candidate risk genes, catechol-o-methyltransferase (COMT) is the only one known associate a functional mutation with increased vulnerability for schizophrenia. COMT is involved in dopamine metabolism, and the val158met polymorphism in \textit{COMT} affects enzymatic activity\(^4\). Carriers for the Met allele have the lowest COMT activity, resulting in reduced dopamine degradation\(^4\). In addition, the Met allele has been associated with a more efficient activation of the prefrontal
cortex and improved cognitive performance in tasks dependent on prefrontal cortical function in healthy and schizophrenic individuals\textsuperscript{423-425}. Mice that overexpress the human COMT-Val variant demonstrated deficits in attentional set-shifting, and impairments in object recognition and working memory, along with diminished stress responses and pain sensitivity\textsuperscript{426}. In contrast, COMT knock-out mice have an increase in dopamine in the frontal cortex and improved working memory, but elevated stress responses and pain sensitivity\textsuperscript{426, 427}. Consequently, these findings indicate that the COMT val158met polymorphism may be involved in abnormal cognitive processing and stress reactivity in diverse clinical disorders, and may influence natural variation in cognition and stress in healthy populations.

The COMT gene is located on the 22q11 locus, which is a region that has been linked to greater schizophrenia risk\textsuperscript{1, 21}. Deletions in this chromosomal region are associated with velocardiofacial syndrome, a disease accompanied with an increased prevalence of psychotic symptoms\textsuperscript{1, 21}. The proline dehydrogenase (PRODH) gene is also located in the 22q11 region. Studies have identified a significant association of PRODH with schizophrenia vulnerability\textsuperscript{428, 429} and the presence of missense mutations in this gene in patients with schizophrenia\textsuperscript{430}. PRODH is involved in the catabolism of proline, which in turn affects glutamate availability and acetylcholine function in the cortex\textsuperscript{431, 432}. Mice with a deficiency in PRODH exhibit aberrant glutamatergic transmission, deficits in sensorimotor gating and associative memory, and exaggerated responses to stress and amphetamine\textsuperscript{433, 434}. Additionally, an epistatic interaction between the PRODH and COMT genes was demonstrated in these mice, as reductions in PRODH resulted in an upregulation of COMT mRNA in the frontal cortex and altered behavioral responses to a COMT inhibitor\textsuperscript{433}. 
Neuregulin-1 (NRG1) was originally identified as a candidate gene following fine-mapping of a locus on chromosome 8p, a region that has been frequently linked to schizophrenia. Numerous studies have since confirmed the association of NRG1 with greater schizophrenia risk, and a variant in the NRG1 promoter region has been associated with decreased cortical activation, the development of psychotic symptoms, and reduced premorbid IQ. NRG1 has a range of roles in the development and function of the nervous system, affecting neuronal migration and differentiation, synapse formation, glial proliferation, synaptic plasticity, and neurotransmitter receptor expression and function. Furthermore, the NRG1 receptor ErbB4 is critically involved in glutamatergic synapse maturation and signaling. Mice with heterozygous deletions in selective domains of NRG1 demonstrate locomotor hyperactivity, altered exploratory behaviors, disrupted sensorimotor gating and LI, and are responsive to antipsychotics. In rodents, locomotor hyperactivity and disrupted LI are considered to be models relevant to the positive symptoms of schizophrenia, corresponding respectively to the psychomotor agitation and loss of LI observed in patients displaying acute psychotic symptoms. Reductions in the ErbB4 receptor similarly induce hyperactivity in mice, as well as impairments in prepulse inhibition and spatial learning and memory. Together these results suggest an important role for NRG1 and ErbB4 in biological mechanisms underlying the positive and cognitive symptoms of schizophrenia. Interestingly, a recent study indicates that NRG1-ErbB4 signaling may contribute to NMDAR hypofunction in schizophrenia, as NRG1-induced activation of ErbB4 was found to be elevated in the prefrontal cortex of schizophrenia patients and increased NRG1 stimulation led to the suppression of NMDAR activation in human cortical tissue.
*Dysbindin* (dystrobrevin binding protein 1) is another susceptibility gene that was initially associated with schizophrenia through linkage to chromosome 6p\(^443\). The positive association of this locus with schizophrenia has been replicated in several subsequent independent studies, some of which show that genetic variation in the dysbindin gene influences general cognitive ability and prefrontal brain function in healthy populations, as well as negative symptoms and cognitive decline in schizophrenia patients\(^444-448\). The functions of dysbindin are not well understood, although growing evidence supports a role in neurotransmitter release, affecting the kinetics, amount, and probability of presynaptic vesicular release and the morphology of vesicles\(^449\). In primary neuronal cultures dysbindin has been shown affect extracellular glutamate release and promote neuronal viability\(^450\). Additionally, dysbindin binds to β-dystrobrevin, a member of the dystrophin protein complex that affects synaptic structure and signaling\(^451\). Studies of the homozygous sandy mice that have a loss in dysbindin protein show behavioral disturbances that include impairments in object recognition memory, social interactions, long-term and spatial working memory\(^452-454\). Pharmacological responses to amphetamine and pain sensitivity are also abnormal in the sandy mice\(^454\). Thus, results demonstrating social withdrawal and cognitive deficits in mice that lack dysbindin protein are consistent with association studies indicating that aberrant dysbindin activity may contribute to negative symptoms and cognitive dysfunction in schizophrenia. Furthermore, the behavioral abnormalities in the sandy mice are surprisingly similar to mice with aberrant NMDAR glycine site function, and this may reflect converging functions of these genes. Indeed, epistatic effects and common protein-protein interactions have been reported for schizophrenia susceptibility genes, suggesting some overlap of common biological processes\(^433, 455, 456\).
Disrupted-in-schizophrenia 1 (*DISC1*) was first identified at the breakpoint of a balanced chromosomal translocation t(1;11) (q42.1; q14.3) which cosegregated in a large Scottish family with schizophrenia and other major psychiatric disorders. Multiple independent studies have since shown association between polymorphisms in *DISC1* and schizophrenia susceptibility in diverse population samples, along with several reports indicating that allelic variation in *DISC1* is associated with abnormal cortical and hippocampal gray matter volume and function, positive symptoms, cognitive impairments, and social anhedonia. *DISC1* plays an important role in CNS development and neuronal functions that includes involvement in neuronal migration and maturation, neurite outgrowth, cytoskeletal function, synaptic transmission, and plasticity. Mice with altered *DISC1* function display phenotypes relevant to schizophrenia and mood disorders. Examination of mice with ENU-induced missense mutations in exon 2 of the *DISC1* gene revealed that a mutation at amino acid position 100 (L100P) resulted in schizophrenia-like behavioral abnormalities that were normalized by typical and atypical antipsychotics, while a mutation at amino acid position 31 (Q31L) produced depressive-like phenotypes that were reversible by antidepressant treatment. Both the L100P and Q31L mutant mice demonstrated disrupted LI, working memory impairments, and a decrease in brain volume; however, the L100P mutant mice also demonstrated locomotor hyperactivity and severe sensorimotor gating deficits, while the Q31L showed additional phenotypes related to depression, anhedonia, and social withdrawal. Expression of truncated DISC1 protein on a background of endogenous mouse DISC1 protein led to numerous structural, cellular, and behavioral perturbations. These included an enlargement of the lateral ventricles, reductions in cortical thickness, neurite outgrowth, parvalbumin GABAergic neurons in the
hippocampus and cortex, as well as hyperlocomotion, sensorimotor gating deficits, spatial memory impairments, and augmented depressive-like behaviors\textsuperscript{464-466}. Furthermore, expression of a C-terminal portion of DISC1 in mice at postnatal day 7, but not in adulthood, produced social impairments, depressive-like behaviors, spatial working memory deficits, and decreased dendritic branching in the hippocampus\textsuperscript{461}. Overall, studies in genetic mouse models investigating the effects of altered DISC1 function indicate a broad spectrum of abnormalities pertinent to schizophrenia and affective disorders, suggesting that DISC1 is a major component in a multidimensional risk pathway for psychiatric illness.

Several other candidate genes involved in schizophrenia risk have been identified and described in recent reviews\textsuperscript{1, 21}. For some genes, preliminary characterization of genetic mouse models has indicated phenotypes that are relevant to schizophrenia and other psychiatric conditions. For example, deletion of the metabotropic glutamate receptor 3 (GRM3) gene in mice affects vulnerability to neurotoxic insults\textsuperscript{467}, alters the expression of glutamate receptors and transporters in the hippocampus\textsuperscript{468}, and augments the sensitivity to dopamine D2 receptor agonists in striatal tissue\textsuperscript{469}. Assessment of mice with a neuronal PAS domain 3 (NPAS3) deficiency reveals abnormalities in brain development, neurosignaling, and behavior, including enlarged ventricles, locomotor hyperactivity, disrupted sensorimotor gating, and resistance to antipsychotic medications\textsuperscript{470}. Further evaluation of these and other current models will be valuable to our understanding of the function of these genes and their importance to the pathophysiology of schizophrenia.

A lack of overt behavioral phenotypes has been observed in some candidate gene mouse models. Regulator of G-protein signaling 4 (RGS4) has been associated with schizophrenia in multiple studies\textsuperscript{21} and decreased RGS4 transcripts have been reported in a
number of cortical regions in schizophrenia patients\textsuperscript{471}. However, mice with a RGS4 deletion show intact locomotor activity, prepulse inhibition, and working memory\textsuperscript{472}.

Absence of behavioral abnormalities in the RGS4 null mice and other candidate gene mouse models may be related to developmental compensatory effects, the obscuring of phenotypes by a mixed genetic background, procedural limitations, or a lack of involvement of the gene in the assessed behaviors or schizophrenia. Hence, it is crucial that behavioral phenotyping strategies take into account the influences of sex and background strain, in addition to undertaking a comprehensive approach that assesses multiple endophenotypes related to psychiatric illness and controls for sensory, motor, visual, olfactory, and other functions that may influence behavioral performance. Since conventional knockout and certain transgenic technologies induce gene mutations from conception, the lifelong absence of a gene will often induce compensatory processes and developmental defects that contribute to the adult phenotype in tandem with the targeted mutation. Consequently, mutations that have regional and temporal specificity may be of interest, as this strategy may notably limit widespread compensatory changes. Inducible and region-specific mutations will also allow the study of alterations that more closely reflect the subtle perturbations found in schizophrenia and other psychiatric disorders. Furthermore, assessment of mice with single nucleotide substitutions similar to those reported in schizophrenia will be required to study the effects of variations at risk alleles. Many of the polymorphisms related to schizophrenia affect regulatory elements of genes, and the examination of animal models with these genetic abnormalities will help clarify the effect these mutations have on gene processing and regulation as well as their role in the more complex molecular processes involved in schizophrenia. An extension to this approach is to eliminate the mouse gene of interest and
replace it with the human ortholog (knock-in) containing the risk variant, in an effort to better predict the effects of the polymorphism in humans. Since schizophrenia likely arises from the simultaneous disruption of several genes, compound mutant mice in which several candidate genes are targeted may offer the greatest potential for future genetically modified mice. Partial gain- or loss-of-function mutations in several risk genes within the same animal would most closely replicate the etiological mechanism of schizophrenia and would be highly valuable for the development of novel therapeutic interventions.

The future of NMDAR glycine site models and their application in the treatment of schizophrenia

Substantial behavioral characterization of genetic models of altered NMDAR glycine site function and D-serine modulatory enzymes has been completed (Table 7.1)\textsuperscript{140, 280, 372, 401}. However, little is known on the effects of reduced NMDAR glycine site activation on structural and cellular changes relevant to schizophrenia. Structural abnormalities in schizophrenia include lateral and third ventricular enlargement and volume reductions in certain structures like the hippocampus, frontal lobe (prefrontal and orbitofrontal), and superior temporal gyrus\textsuperscript{21, 473}. In addition to cytoarchitectural abnormalities, disarrayed neuronal arrangements, and ectopic expression of neurons, histological observations have indicated reductions in dendritic spines and arborizations, decreased interneuron density, and altered expression of myelination genes along with reductions in the number and function of oligodendrocytes\textsuperscript{21, 473}. Activation of NMDARs stimulates neuronal differentiation, axonal growth, and dendritic spine proliferation\textsuperscript{474}. NMDARs can also regulate neuronal migration and are involved in activity-dependent modification of synaptic connections in the
developing brain\textsuperscript{474, 475}. Consequently, investigations to determine the structural and cellular effects of genetic modification of the NMDAR glycine site, Srr, and DAO may reveal schizophrenia-like neuropathology. Indeed, a functional genomic analysis of the \textit{Srr}\textsuperscript{Y269*} mice revealed differential expression of several genes involved in myelination and neurodevelopment, as well as genes implicated in schizophrenia and cognitive ability\textsuperscript{140}.

Considering the importance of the glycine site in regulating NMDAR-mediated neurotransmission, it may be possible that abnormal modulation of D-serine/glycine availability induces disturbances in other systems that interact with the NMDAR, including the dopaminergic and GABAergic pathways. Altered responses to compounds that stimulate dopamine release have been demonstrated in DAO and GlyT-1 mutant mice\textsuperscript{397, 403}, though more a extensive analysis of dopaminergic changes is required. Similarly, it would be interesting to examine whether GABAergic changes are present in these mice, and initial experiments may include measures of GAD67 and parvalbumin expression, as well as electrophysiological recordings of inhibitory postsynaptic currents in hippocampal and cortical regions. Further characterization of the molecular mechanisms involved in the complex interaction between the dopaminergic, GABAergic, and glutamatergic pathways will benefit our understanding of normal physiology and the contributions of these systems to disease states.

As schizophrenia involves both genetic and environmental factors, investigation of a two-hit model that combines neurodevelopmental disruptions with a genetic modification affecting NMDAR glycine site occupancy would be of value. Early exposure to stress, social isolation, or maternal infection models all produce schizophrenia-like phenotypes in adult mice\textsuperscript{476-479} that may be further exacerbated by genetic manipulation of the NMDAR glycine
site or D-serine modulatory enzymes. In support, maternal infection models have been shown to enhance sensitivity to NMDAR antagonists, decrease hippocampal NR1 expression, and augment brain levels of the endogenous NMDAR glycine site antagonist kynurenic acid in adult mice\textsuperscript{479, 480}. Prenatal stress also induces long-term alterations in NMDAR subunit expression in prefrontal cortex, hippocampus, and striatum of adult offspring\textsuperscript{481, 482}, and stress, through the actions of glucocorticoid and corticotrophin-releasing hormone, modulates glutamate release and NMDAR activation\textsuperscript{483}. Thus, neurodevelopmental insults produce enduring perturbations in the NMDAR system that may further aggravated by a genetic reduction in NMDAR glycine site occupancy. As a comparison, an additional two-hit model involving genetic perturbation of dopaminergic function may be useful. Alternatively, modification of the environment can also be used to ameliorate existing behavioral disturbances in genetically modified mice. Environmental enrichment has been shown to overcome the diminution of cognitive function in animal models of neurodegeneration\textsuperscript{484}, and may induce similar improvements in models pertinent to schizophrenia. Indeed, environmental enrichment reversed locomotor hyperactivity and sensorimotor gating deficits in mice with deficient phospholipase C-beta1, an enzyme involved cortical development and neuronal plasticity\textsuperscript{485}.

Regarding the future of pharmaceutical interventions involving the NMDAR glycine site, overall findings from preclinical and clinical studies demonstrate apparent beneficial effects, particularly on the negative and cognitive symptoms domains. Though administration of direct agonists, such as D-serine and glycine, are of therapeutic value, such treatments require large doses and exhibit difficulties in penetrating the blood-brain-barrier\textsuperscript{15, 370}. Consequently, agents targeting proteins involved in D-serine metabolism may be a more
effective strategy, providing selective modulation of the NMDAR glycine site44. Inhibition of DAO activity in the brain is of particular interest as it would circumvent any nephrotoxicity associated with the catabolism of high levels of systemic D-serine371. Development of DAO antagonists has recently begun399, 486-488. Intravenous injections of the DAO inhibitor AS057278 were found to readily cross the blood-brain-barrier and enhance D-serine contents in the rat brain399. Chronic administration of AS057278 in mice was shown to normalize PCP-induced deficits in behavioral tasks relevant to the cognitive and positive symptoms of schizophrenia, whereas acute DAO inhibition had limited efficacy399, 488. DAO inhibitors could also be used to further enhance the ameliorative effects of a low dose of exogenous D-serine. Sensorimotor gating deficits induced by MK-801 were reversed by coadministration of D-serine and a DAO antagonist, but not by D-serine treatment alone400. Thus, DAO inhibition in combination with D-serine administration may be a valuable therapeutic approach for the treatment of schizophrenia. Activators of Srr function may also provide beneficial effects, however such compounds have yet to be developed. In sum, agents targeting the NMDAR glycine site are examples of fundamentally novel therapies that may deliver symptomatic improvement with fewer side effects than current antipsychotics.
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