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ORIGINAL INVESTIGATION

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

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Abstract
Rationale 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.
Objective We examined the involvement of the glycine coagonist site on the *N*-methyl-D-aspartate receptor (NMDAR) glycine coagonist site in the modulation of negative and cognitive endophenotypes in mice.
Materials and methods Behavioral phenotypes relevant to schizophrenia were assessed in Grin1^{D481N} mice that have reduced NMDAR glycine affinity.
Results Grin1^{D481N} mutant mice showed abnormally persistent latent inhibition (LI) that was reversed by two agents that enhance NMDAR glycine site function, D-serine (600 mg/kg) and ALX-5407 (1 mg/kg), and by the classical atypical antipsychotic clozapine (3 mg/kg). Similarly, blockade of the NMDAR glycine site with the antagonist L-701,324 (5 mg/kg) induced persistent LI in C57BL6/J mice. In a social affiliations task, Grin1^{D481N} mutant animals showed reduced social approach behaviors that were normalized by D-serine (600 mg/kg). During a nonassociative spatial object recognition task, mutant mice demonstrated impaired reactivity to a spatial change that was reversible by D-serine (300 and 600 mg/kg) and clozapine (0.75 mg/kg). In contrast, responses to social novelty and nonspatial change remained unaffected, indicating that the Grin1^{D481N} mutation induces selective deficits in sociability and spatial discrimination, while leaving intact the ability to react to novelty.
Conclusions 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.

Keywords NMDA receptor · D-serine · Glycine coagonist site · Latent inhibition · Mice · Schizophrenia

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Introduction

Schizophrenia is a severe and debilitating disease that affects multiple domains of higher-order behavior. In addition to the psychotic symptoms, schizophrenia is characterized by profound cognitive deficits that include impairments in attention, working memory, and behavioral flexibility, and by negative symptoms, such as social withdrawal and affective flattening (Lewis and Gonzalez-Burgos 2006; Ross et al. 2006). Current antipsychotics have limited success in treating the negative and cognitive symptoms of this disorder (Lewis and Gonzalez-Burgos 2006; Murphy et al. 2006). Consequently, significant efforts have been made to ameliorate treatments and to further the understanding on the underlying pathophysiological mechanisms.

The *N*-methyl-D-aspartate receptor (NMDAR) hypofunction model proposes that aberrant NMDAR-mediated transmission may be involved in schizophrenia pathophys-

iology (Coyle 2006). Blockade of the NMDAR with noncompetitive antagonists like phencyclidine have been shown to produce and exacerbate positive, negative, and cognitive schizophrenic symptoms (Javitt and Zukin 1991; Krystal et al. 1994). Moreover, genetic linkage studies have identified a number of susceptibility genes that modulate NMDAR function (Chumakov et al. 2002; Ross et al. 2006). Decreased in vivo hippocampal NMDAR binding and reduced plasma levels of endogenous NMDAR agonists have been reported in drug-naïve schizophrenic patients (Millan 2005; Pilowsky et al. 2006). These observations indicate that diminished NMDAR activity may have a role in schizophrenia and suggest that enhanced activity of the receptor may be therapeutic. Indeed, several clinical studies have shown symptomatic improvements with drugs that directly and indirectly activate NMDAR, including D-serine, which is a highly selective and potent agonist for the NMDA-NR1 glycine site (Coyle 2006; Matsui et al. 1995; Millan 2005; Tsai et al. 1998).

Preclinical models can further the understanding of schizophrenia pathophysiology and pharmacotherapy. Latent inhibition (LI) is a prevalent animal model in schizophrenia research and refers to the process whereby previous non-reinforced exposure to a stimulus affects subsequent learning of new associations with this stimulus (Moser et al. 2000; Gal et al. 2005). 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 (Moser et al. 2000; Weiner 2003). LI is disrupted or absent in humans and rodents given amphetamine and in schizophrenics exhibiting acute positive symptoms (Moser et al. 2000; Rascle et al. 2001; Weiner 2003). Alternatively, the NMDAR antagonist MK-801 can induce abnormally persistent LI in rodents (Gaisler-Salomon and Weiner 2003; Lipina et al. 2005). Perseverative behaviors are associated with the negative symptoms of schizophrenia (Berman et al. 1997; Capleton 1996), and persistent LI correlates with the severity of negative symptoms (Cohen et al. 2004; Rascle et al. 2001). Thus, LI is proposed to model both the positive (disrupted LI) and negative (persistent LI) symptoms, and involves the information-processing abilities affected in schizophrenia (Moser et al. 2000; Weiner 2003).

In the cluster of negative symptoms of schizophrenia, social dysfunction is an important component, beginning in the premorbid stages and continuing on chronically throughout the entire course of the illness (Ellenbroek and Cools 2000; Ross et al. 2006). Cognitive disturbances in schizophrenia have a similar pattern of emergence (Lewis and Gonzalez-Burgos 2006; Ross et al. 2006), and deficits in attention, spatial organization, and visuospatial abilities have been reported to be correlated with social functioning (Cornblatt and Keilp 1994; Dickerson et al. 1996). In animals, measurements of social encounters have served as a heuristic model for the social deficits observed in schizophrenics (Ellenbroek and Cools 2000), while cognitive visuospatial abilities can be investigated using a spatial object recognition procedure that evaluates the reaction to a spatial change (Mandillo et al. 2003; Roullet et al. 1996). Pharmacological studies in rodents have determined prominent impairments in social interactions and spatial recognition tasks after transient NMDAR blockade (Boulay et al. 2004; Roullet et al. 1996; Sams-Dodd 1996). 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 fivefold decrease in NMDAR glycine affinity due to a point mutation (aspartate to asparagine substitution at amino acid 481) in their NR1 glycine binding site (Kew et al. 2000). 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 (Duffy et al. 2007; Kew et al. 2000). 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. 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.

Materials and methods

Animals

Grin1^{D481N} mice were generated by site-directed mutagenesis and homologous recombination (Kew et al. 2000) and derived from founders generously provided by Dr. M. Pauly-Evers, Hoffman-La Roche (Basel, Switzerland). These mice were backcrossed 11 generations onto the C57BL/6J strain and then bred from heterozygous intercrosses in the animal colony at the Samuel Lunenfeld Research Institute, Toronto, Canada. 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 before testing.

Groups of three to five littermates were housed in filtered polycarbonated cages, under controlled temperature (20±1°C), lighting (lights on 0700–1900), and humidity

(50–60%). The animals were given ad libitum sterile food and water, except in LI and olfactory experiments. Behavioral testing was done between 0900 and 1600 hours on experimentally naïve 12- to 16-week-old mice. Experiments were randomized with regard to day and drug treatment, and sex-balanced, except where stated. Experimenters were blind to genotype. The experimental equipment was cleaned with 70% ethanol between each subject. 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.

Latent inhibition

LI experiments were conducted in three chambers (MED Associates, VT, USA), as described previously (Lipina et al. 2005). Mice were weighed and water was removed from home cages 24 h before the start of the LI procedure. Throughout the experiment, weights were monitored to ensure that the 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 two or four 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 that 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 $A/(A+B)$. LI 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 (Brodkin et al. 2004; Moy et al. 2004). The apparatus for this test consisted of a clear Plexiglas box (53 cm length × 25.6 cm width × 23 cm height) divided into three interconnected chambers (outer chambers = 19.5 cm length, central chamber = 13 cm length). The outer chambers were identical to each other and divided from the central chamber by clear Plexiglas partitions (7.3 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 this experiment. The stranger mice used were age-matched, male C57BL/6J mice (The Jackson Laboratory, ME, USA) that had never bred or 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 an event-recording program (The Observer 5.0 by Noldus Information Technology, 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 limited to 5 min to avoid acclimatization to stranger 2. All other parameters and measures were as described above in the sociability phase.

An additional experiment was performed in which there were three sessions. Sessions 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 (Wersinger et al. 2002). 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, IN, 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 (maximum 10 min).

Spatial and nonspatial 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 horizontal and vertical movements (model 7420/7430; Ugo Basile, Italy). The five objects used in this task were: a green plastic box (7.3×5.2×3.8 cm), a brown polypropylene cylinder (7 cm height, 6.1 cm diameter), a blue Pyrex glass cone (6 cm height, 7 cm diameter), a white ceramic semisphere (5.5 cm height, 7.5 cm diameter), and a multicolored plastic cube (5.5 cm). Animal behavior was recorded and analyzed using The Observer 5.0 (Noldus Information Technology, The Netherlands).

The testing procedure was adapted from previously described protocols (Frick and Gresack 2003; Mandillo et al. 2003; Roullet et al. 1996). Only male mice were used, as sex differences have been reported for spatial novelty responses in C57BL/6J mice (Frick and Gresack 2003). On test day, each mouse was individually placed in the center of the empty arena for a 5-min session (S1), and horizontal and vertical 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 four continuous 5-min sessions (habituation phase, S2–S5). Habituation to object exploration was measured by recording the time spent exploring the objects across sessions S2–S5. 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 these sessions (S2–S5) and in subsequent sessions. At the end of S5, 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, S6). Reaction to a spatial change was assessed by comparing the mean time spent exploring the displaced (DO) and nondisplaced (NDO) object categories within S5 and S6 and between S5 and S6.

Reactivity to a nonspatial change was also examined. Directly after S6, 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, S7). Measurements were taken as described for S6, 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).

Vision was verified using the visual placing test (Wersinger et al. 2002). 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.

Drugs

D-serine (Sigma, Canada) was dissolved in a saline (0.9% NaCl) solution. ALX-5407 ((*R*)-*N*-[3-(4′-fluorophenyl)-3(4′-phenylphenoxy)propyl]sarcosine hydrochloride; Sigma, Canada) was dissolved in 75% ddH_2O/25% 2-hydroxypropyl-β-cyclodextrin, and pH was adjusted to approximately 6 using 1 N NaOH. Clozapine (Tocris, USA) and Ro 25-6981 (Sigma, Canada) were dissolved in 0.9% NaCl with 0.3% Tween. L-701,324 (7-chloro-4-hydroxy-3(3-phenoxy)phenylquinoline-2-(*H*)-one; Tocris, USA) was dissolved in a solvent containing 25% polyethyleneglycol-300 and slightly alkalinized with 1 N NaOH. D-serine (300 or 600 mg/kg) and Ro 25-6981 (5 mg/kg) were injected subcutaneously. ALX-5407 (1 mg/kg), clozapine (0.75 or 3 mg/kg), and L-701,324 (5 mg/kg) were injected intraperitoneally. All drugs were administered at volume of 10 ml/kg. Injection–testing intervals were 20 min for D-

serine, 120 min for ALX-5407, 45 min for L-701,324, and 30 min for clozapine and Ro 25-6981. Drug doses were chosen based on previous work (Duffy et al. 2007; Lipina et al. 2005), pilot experiments, and other behavioral studies (Depoortere et al. 1999; Gleason and Shannon, 1997; Karcz-Kubicha et al. 1999; Mohn et al. 1999). In LI, all drugs were given before the start of the preexposure and conditioning phases.

Statistical analysis

Statistical analyses were performed using Statistica (Statsoft, OK, USA). Behavioral data were analyzed using 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.

Results

Latent Inhibition in Grin1^{D481N} mutant mice

LI after two or four conditioning trials (CS-US) was measured in the Grin1^{D481N} mice. No differences were found in the drinking performance of mutant mice during the pretraining days, as measured by the latency to the first lick (wild-type=135.09±34.57 s; mutant=127.06±34.40 s) and the number of licks (wild-type=526.32±36.73 s; mutant=476.48±20.43 s). On test day, the time taken to complete lick 50–75 (A period) before CS onset did not differ between genotype, drug treatment, or preexposure group (2CS-US group=6.66±1.81 s, 4CS-US drug-naïve group=7.64±1.20 s, 4CS-US drug-treated group=5.10±0.53 s). Male and female suppression ratios were combined for each preexposure condition, as no gender effects were observed. Two mice were excluded because of a failure to drink on the baseline day (mutant in the 2CS-US group, mutant in the 4CS-US drug-treated group).

2CS-US As depicted in Fig. 1a, LI was present in both wild-type and mutant mice given 2CS-US (main effect of preexposure: $F_{1,28}=24.5$, $p<0.001$), as there was a difference between the PE and NPE groups within each genotype ($p<0.01$).

4CS-US LI was also assessed in wild-type and mutant mice given 4CS-US pairings (Fig. 1b). A significant main effect of preexposure ($F_{1,32}=5.87$, $p<0.03$) was found. In contrast to wild-type animals, Grin1^{D481N} mutant mice continued to show LI despite the increase in conditioning trials ($p<0.009$). Aberrantly persistent LI in mutant animals could be reversed by the direct NMDAR glycine site agonist D-serine (Matsui et al. 1995), the high affinity inhibitor of the glycine transporter type 1 ALX-5407 (Atkinson et al. 2001), and the atypical antipsychotic clozapine (Fig. 1c). Enduring LI was demonstrated in vehicle-injected Grin1^{D481N} mutant mice ($p<0.03$). However, in mutant mice given D-serine, ALX-5407, or clozapine, LI was not present, similar to wild-type mice given 4CS-US pairings. In the ALX-5407 and clozapine groups, the NPE ratio was significantly elevated compared to the NPE ratio of vehicle-treated mutants ($p<0.03$).

Latent Inhibition 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

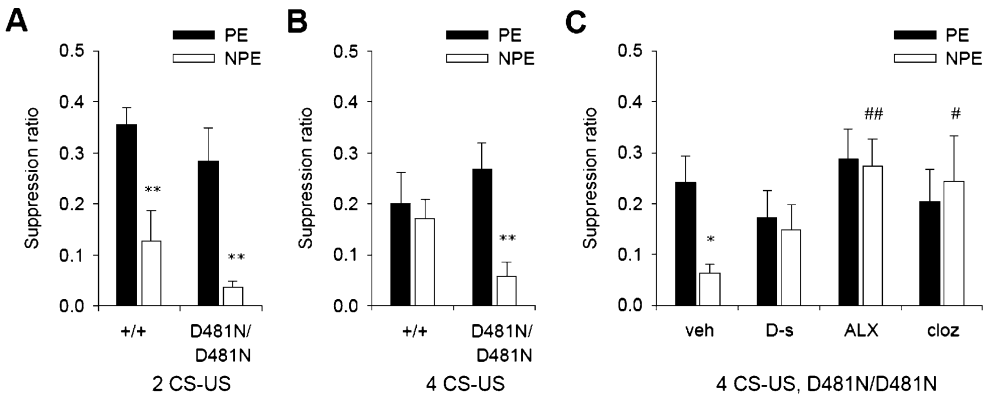

Fig. 1 LI in mice with reduced NMDAR glycine site function. Mean suppression ratios of preexposed (*PE*) and nonpreexposed (*NPE*) wild-type (+/+) and Grin1^{D481N} mutant (*D481N/D481N*) mice given 2CS-US (**a**) or 4CS-US (**b**) pairings. **c** Mean suppression ratios of mutant mice that received 4CS-US pairings and treatments of vehicle (*veh*), D-serine (600 mg/kg; *D-s*), ALX-5407 (1 mg/kg; *ALX*), or clozapine (3 mg/kg; *cloz*). $n=7$–10 per group; *$p<0.05$, **$p<0.01$—NPE 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

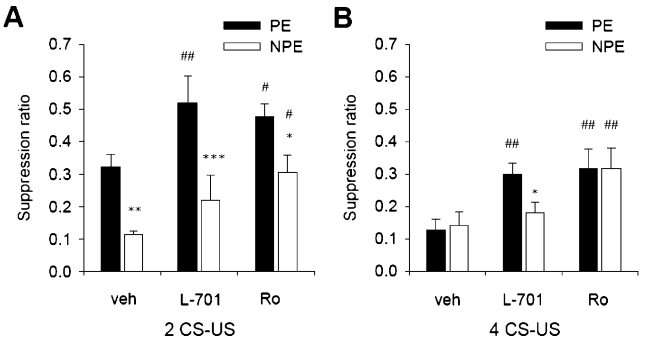

Fig. 2 LI in C57BL/6 mice treated with the NMDAR glycine site antagonist L-701,324 or the NMDA-NR2B receptor inhibitor Ro 25-6981. Mean suppression ratios of preexposed (*PE*) and nonpreexposed (*NPE*) C57BL/6 mice administered vehicle (*veh*), L-701,324 (5 mg/kg; *L-701*), or Ro 25-6981 (5 mg/kg; *Ro*). Drug effects were examined in animals given 2CS-US (**a**) or 4CS-US (**b**) pairings. n=7–15 per group; *p<0.05, **p<0.01, ***p<0.001—NPE compared to PE score within each drug treatment; #p<0.05, ##p<0.01—compared to vehicle-treated C57BL/6J mice with the same preexposure condition (*PE* or *NPE*)

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 (Duffy et al. 2007; Higgins et al. 2003; Sotres-Bayon et al. 2007). To assess the effects of the antagonists under conditions that produce LI in vehicle-treated mice, a 2CS-US protocol was used. To estimate the ability of the drugs to influence persistent LI, a 4CS-US protocol was applied. No differences were demonstrated in drug treatment or preexposure groups in the time taken to consume 50–75 licks before CS onset (2CS-US group=8.48±1.52 s, 4CS-US group=9.71±1.65 s).

2CS-US Initially, C57BL/6J mice treated with vehicle, L-701,324, or Ro 25-6981 were given 2CS-US pairings (Fig. 2a). There was a significant main effect of preexposure ($F_{1,44}$=28.2, p<0.001) and of drug treatment ($F_{2,44}$=7.29, p<0.002), and LI was present in all treatment groups (p<0.03). Also, animals given Ro 25-6981 had a significantly higher NPE ratio compared to vehicle-treated mice (p<0.02), suggesting that though LI is present in Ro 25-6981-treated mice, this group may also exhibit a minor reduction in associative learning.

4CS-US As shown in Fig. 2b, LI was not demonstrated in vehicle-treated C57BL/6J mice given 4CS-US pairings. However, a main effect of drug treatment ($F_{2,62}$=8.39, p< 0.001) was determined because LI was present in animals given L-701,324 (p<0.03). The PE score of L-701,324-treated mice was significantly elevated compared to the PE score of vehicle-treated mice (p<0.003), 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 (Duffy et al. 2007). 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.

Social affiliations

To further investigate the role of reduced glycine affinity in negative-like symptoms of schizophrenia, the social approach behaviors of the Grin1^{D481N} mice were assessed. Performance of wild-type and mutant mice in a test of sociability is shown in Fig. 3a. Unlike wild-type mice, mutant animals did not prefer the chamber containing an unfamiliar conspecific mouse (stranger 1) over the empty chamber (main effect of genotype: $F_{1,25}$=12.81, p<0.002). Conversely, wild-type and mutant mice performed similarly in the social novelty phase (Fig. 3b). A main effect of apparatus side ($F_{1,25}$=40.20, 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). In addition, 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 (Fig. 3c and d). In the social novelty phase, there was a significant main effect of apparatus side ($F_{1,25}$=18.00, 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^{D481N} mutant mice was further confirmed in an experiment involving a second sociability phase (Fig. 3e). A significant genotype×apparatus side interaction ($F_{1,14}$=12.85, p< 0.003) was found. In sessions 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<0.002).

The effectiveness of D-serine to normalize social approach deficits in Grin1^{D481N} mutant mice was assessed and compared to clozapine (Fig. 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: $F_{2,54}$=5.69, p<0.006, apparatus side: $F_{1,54}$= 34.75, p<0.001, and genotype×drug treatment×apparatus side interaction: $F_{2,54}$=4.06, p<0.03). A significant preference for the chamber containing the stranger mouse was seen in wild-types treated with vehicle, D-serine, or

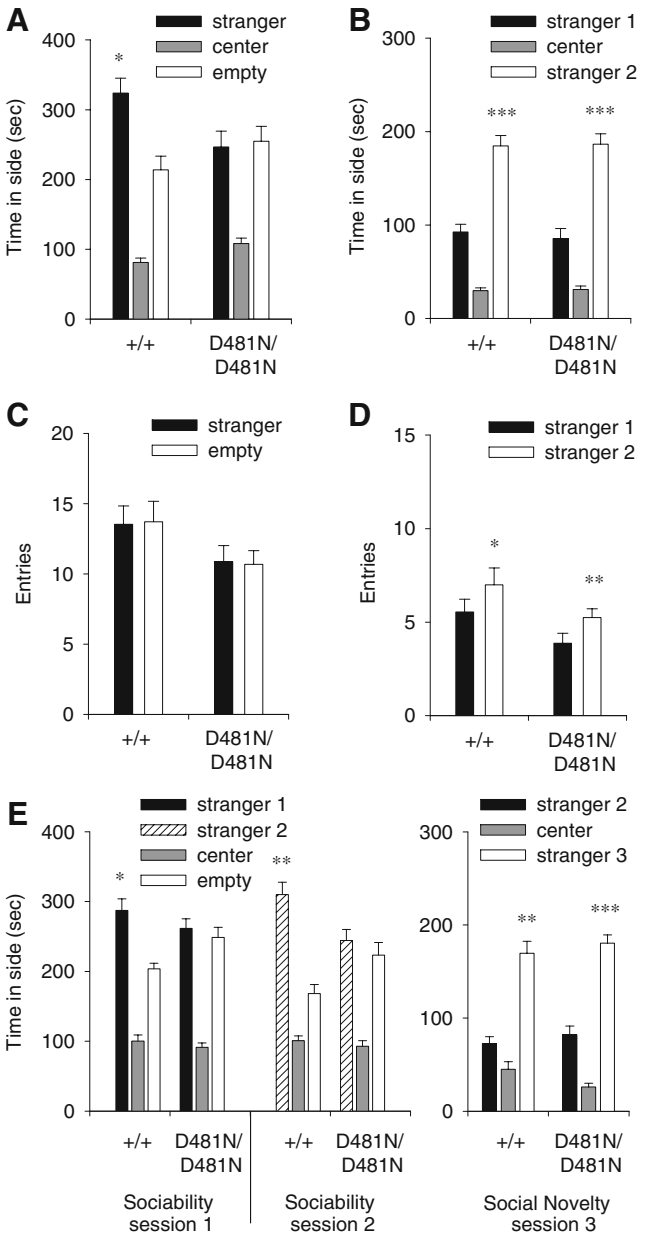

Fig. 3 Social approach behaviors in mice with diminished NMDAR glycine affinity. Wild-type (+/+) and Grin1^{D481N} mutant (*D481N/D481N*) mice were assessed in a social affiliations task. **a** Mean time (s) spent in a chamber containing a stranger mouse, a central chamber, and a chamber with an empty cage in the sociability phase. **b** Average time (s) 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 phase. Number of chamber entries during the sociability (**c**) and social novelty (**d**) phase. **e** Mean time (s) spent with stranger 1 (session 1) or stranger 2 (session 2), a central chamber, and a chamber with an empty cage during the sociability phases (*left panel*). Mean time (s) 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 phase (session 3; *right panel*). n=11 (**a**–**d**) or 8 (**e**) wild-types and 16 (**a**–**d**) or 8 (**e**) mutants; *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

clozapine (p<0.05) and in D-serine-treated mutants (p< 0.05), but not in mutants injected with vehicle (p>0.05) or clozapine (trend p=0.06).

The exploratory activity of vehicle-treated mutants did not differ from vehicle-treated wild-types, as demonstrated in Fig. 4b by the number of entries into the stranger and empty side. However, a reduction in the number of entries into each chamber was observed in wild-types and mutants injected with D-serine or clozapine. There was a main effect of drug treatment ($F_{2,54}$=16.22, p<0.001) and apparatus side ($F_{1,54}$=16.48, p<0.001). D-serine-or clozapine-treated mice displayed fewer entries into the stranger and empty side (p<0.05), and mutants given D-serine or clozapine favored the side with the stranger mouse (p<0.04).

The deficit in social behavior of the Grin1^{D481N} mutants 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-types treated with vehicle=143.05±38.57 s, 600 mg/kg D-serine=108.30±29.68 s, 0.75 mg/kg clozapine= 112.00±10.69 s; mutants treated with vehicle=75.79±

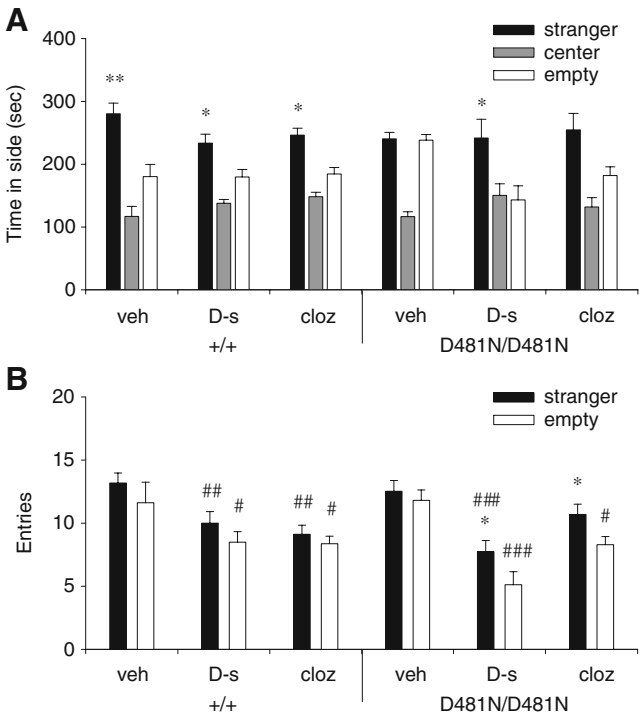

Fig. 4 Social approach behaviors in Grin1^{D481N} mutant mice treated with D-serine or clozapine. Wild-type (+/+) and Grin1^{D481N} mutant (*D481N/D481N*) animals injected with vehicle (*veh*), D-serine (600 mg/kg; *D-s*), or clozapine (0.75 mg/kg; *cloz*) were assessed in a test of sociability. **a** Mean time (s) spent in a chamber containing a stranger mouse, a central chamber, and a chamber with an empty cage. **b** Number of stranger or empty chamber entries. 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

10.33 s, 600 mg/kg D-serine=89.04±23.17 s, 0.75 mg/kg clozapine=95.88±11.28 s).

Spatial and nonspatial recognition

Since cognitive deficits are a core feature of schizophrenia and include impairments in spatial recognition (Lewis and Gonzalez-Burgos 2006; Ross et al. 2006), the 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 horizontal and vertical locomotor activity were detected (data not shown). The duration of locomotor activity in the object habituation sessions (S2–S5) and in the subsequent sessions of spatial change (S6) and nonspatial change (S7) also did not differ between wild-type and mutant mice (Fig. 5a).

As represented in Fig. 5b, wild-type and mutant mice progressively reduced their exploration of all four objects across habituation sessions similarly (main effect of session: $F_{3,42}$=12.90, p<0.001). 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 (data not shown).

The exploration time of the displaced objects (DO) and nondisplaced objects (NDO) was evaluated in the last habituation session (S5) and in the spatial change session (S6) (Fig. 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 ($F_{1,14}$=10.41, p<0.007), object category ($F_{1,14}$=33.72, p<0.001), and genotype×object category interaction ($F_{1,14}$=40.28, 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<0.04). Also, the amount of time spent with the NDO in S6 compared to S5 did not differ in wild-types, but was significantly increased in mutants

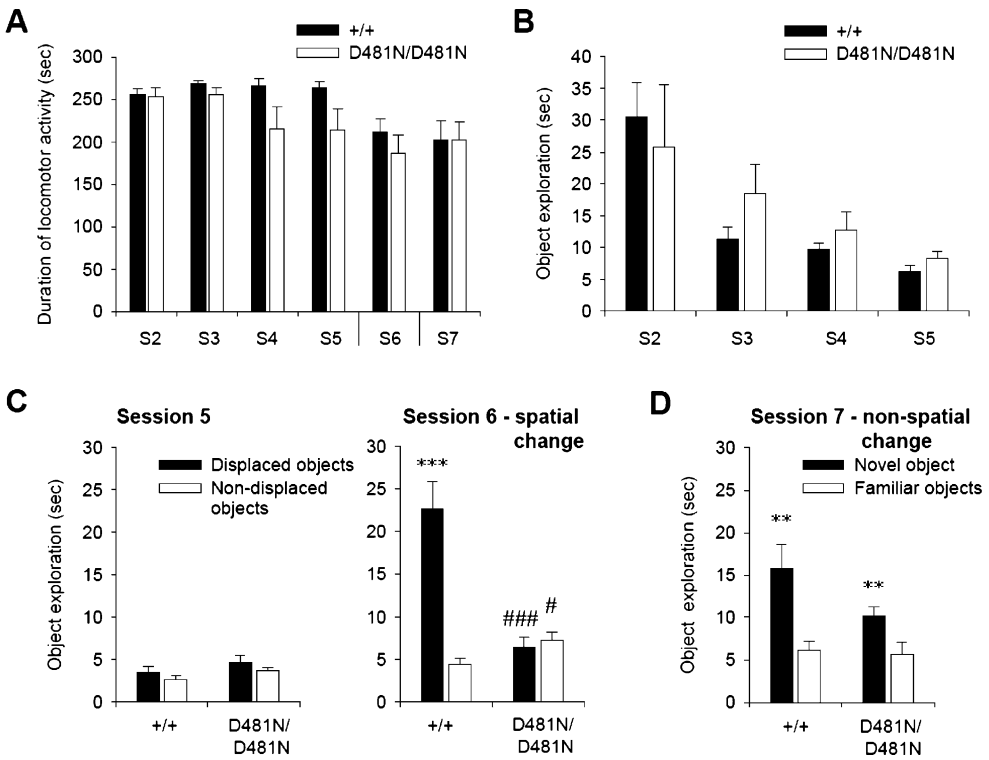

Fig. 5 Spatial and nonspatial object recognition in mice with reduced NMDAR glycine site occupancy. **a** Mean duration (s) of locomotor activity in the habituation phase (*S2–S5*), spatial change phase (*S6*), and the nonspatial change phase (*S7*) for wild-type (+/+) and Grin1^{D481N} mutant (*D481N/D481N*) mice. **b** Mean duration (s) of object exploration in the habituation phase. **c** Average time (s) spent exploring the displaced and nondisplaced objects for wild-type and mutant animals during the last habituation session (session 5; *left panel*) and the spatial change session (session 6; *right panel*). **d** Average time (s) spent exploring the novel item and three familiar objects in the nonspatial change session (session 7). n=8 wild-types and 8 mutants; **p<0.01, ***p<0.001—compared to the time spent exploring the nondisplaced or familiar objects within each genotype; #p<0.05, ###p<0.001—compared to the time the wild-types spent exploring the same object category

Table 1 Locomotor activity in the drug-treated groups during S1 of the spatial recognition experiment

	Horizontal	Vertical
$Grin1^{+/+}$		
Vehicle	256.55±37.87	22.00±5.46
D-serine 300	315.38±28.60	25.88±3.60
D-serine 600	313.00±31.68	19.50±4.59
Clozapine	340.90±32.18	48.00±6.90***
$Grin1^{D481N/D481N}$		
Vehicle	283.44±29.48	22.67±3.93
D-serine 300	246.71±29.37	8.71±2.19
D-serine 600	320.00±20.03	11.13±2.22
Clozapine	374.88±69.33	36.50±6.17*

Data are expressed as the number of beam breaks±SEM.
*$p \leq 0.05$, *** $p < 0.001$ compared to the vehicle-treated group within each genotype (post hoc LSD test, ANOVA)

($p < 0.005$). This indicates that in wild-types, increased exploration was targeted to the objects that underwent a spatial change, whereas in mutant mice, exploratory activity nonspecifically 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 nonspatial change (Fig. 5d). There was a main effect of object category ($F_{1,14}=30.03$, $p<0.001$) related to the preferential exploration of the novel object (NO) compared to the three familiar objects (FOs) by wild-type and mutant animals ($p<0.005$). 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 600-mg/kg D-serine dosage to limit the possibility of exploratory changes. Table 1 presents the locomotor activity in session 1 for wild-type and mutant mice treated with vehicle, D-serine, or clozapine. No differences in horizontal activity were exhibited, but for vertical activity, a significant main effect of drug treatment was determined ($F_{3,59}=12.73$, $p<0.001$) because of an elevation in clozapine-treated wild-type and mutant animals ($p \leq 0.05$). Duration of locomotor activity in the sessions of object habituation (S2–S5) and spatial change (S6) did not differ between any group (Fig. 6a).

Figure 6b demonstrates a comparable gradual decrease in the exploration time of all four objects during the habituation phase (main effect of session: $F_{3,177}=75.30$, $p<0.001$). An analysis of the time spent investigating each individual object indicated that exploration again did not favor any object (data not shown).

Deficient reactivity to a spatial change was improved by D-serine and clozapine treatments in $Grin1^{D481N}$ mutant mice (Fig. 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 mutants, reacted by selectively exploring the DO more than the NDO. There was a significant main effect of object category ($F_{1,65}=131.70$, $p<0.001$) and genotype × drug treatment × object category interaction ($F_{3,65}=7.40$, $p<0.001$). The exploration time of the DO and NDO differed in vehicle-, D-serine-, or clozapine-injected wild-types ($p<0.02$) and in mutants given D-serine or clozapine ($p<0.02$), but not in vehicle-injected mutants. 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 mutants which investigated the NDO more in S6 than in S5 ($p<0.007$). Also, the visual placing test confirmed that visual acuity was normal in all genotype and drug treatment groups (data not shown).

Discussion

In this study, we demonstrated that a reduction in NMDAR glycine affinity in mice induced abnormally persistent LI, 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, and increased motor activity, stereotypy, and sensitivity to amphetamine (Bickel et al. 2007; Duncan et al. 2004; Mohn et al. 1999; Moy et al. 2006). 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 (Ballard et al. 2002; Single et al. 2000). 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.

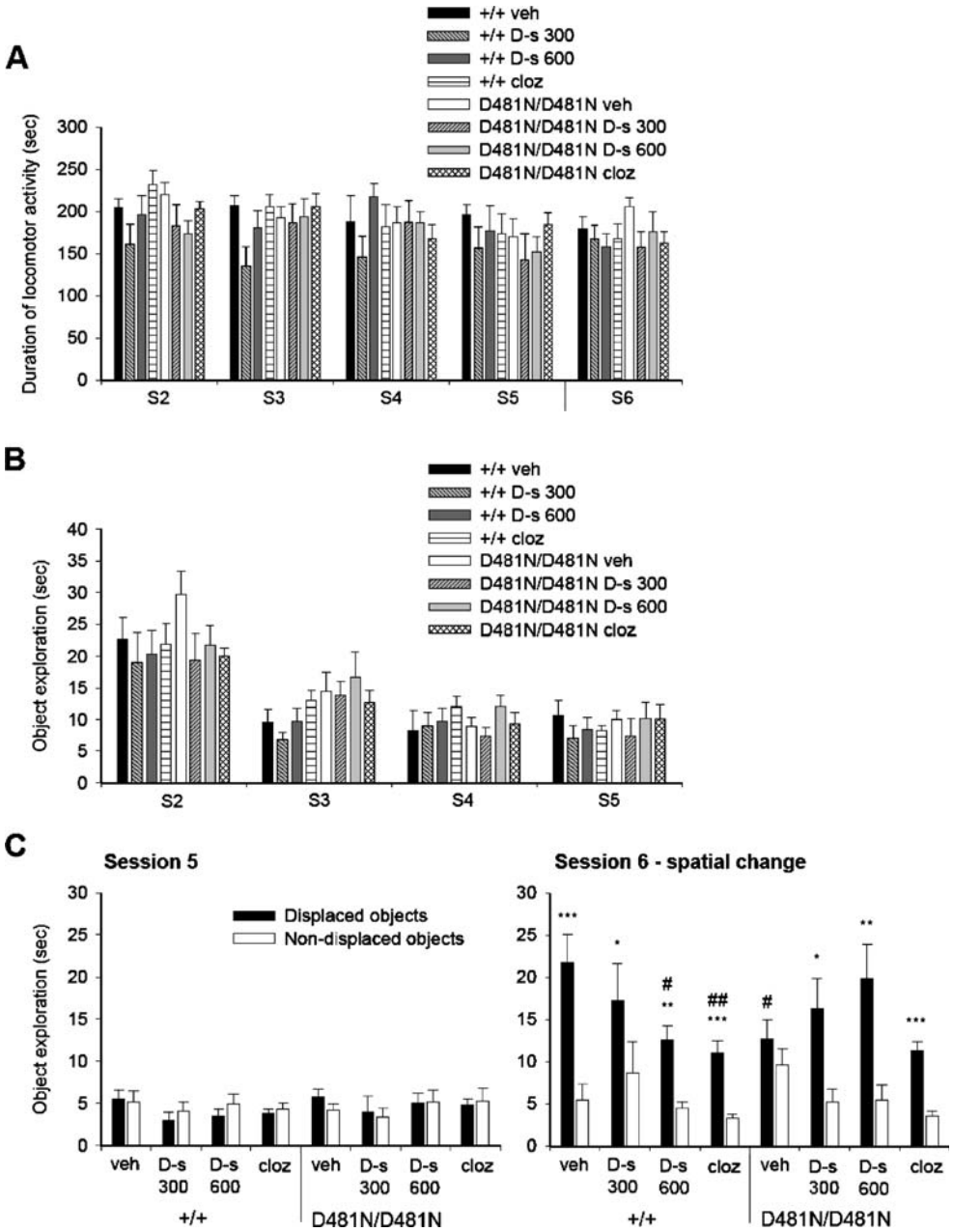

Fig. 6 Spatial object recognition in Grin1^{D481N} mutant mice treated with D-serine or clozapine. Wild-type (+/+) and Grin1^{D481N} mutant (*D481N/D481N*) animals were given either vehicle (*veh*), D-serine (300 or 600 mg/kg; *D-s*), or clozapine (0.75 mg/kg; *cloz*) treatments and reactivity to a spatial change was examined. **a** Mean duration (s) of locomotor activity in the habituation phase (*S2–S5*) and spatial change phase (*S6*). **b** Mean duration (s) of object exploration in the habituation phase. **c** Average time (s) spent exploring the displaced and nondisplaced objects during the last habituation session (session 5; *left panel*) and the spatial change session (session 6; *right panel*). n=7–14 per group; *p<0.05, **p<0.01, ***p<0.001—compared to time spent exploring the nondisplaced 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

Although genetic linkage studies have not associated polymorphisms in the NMDAR to schizophrenia (Martucci et al. 2003; Rice et al. 2001), 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, which are involved in D-serine catabolism, and the D-serine synthesis enzyme, serine racemase (Chumakov et al. 2002; Goltsov et al. 2006; Morita et al. 2006; Schumacher et al. 2004). Morita et al. (2006) 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 (Hashimoto et al. 2003; 2005), while endogenous antagonists for the NMDAR glycine site were observed to be increased in the CSF and cortex (Millan 2005). Therefore, the decreased occupancy and activation of the NMDAR glycine site in the Grin1^{D481N} 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 Grin1^{D481N} mutant animals given 2CS-US trials. This is in agreement with several LI studies that have demonstrated that low doses of NMDAR antagonists do not abolish LI (Moser et al. 2000; Weiner and Feldon 1992). However,

when experimental conditions were increased to 4CS-US pairings, LI was disrupted in wild-type mice, but persevered in Grin1^{D481N} mutants. 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, because unlike the preexposed wild-types, mutants 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 (Berman et al. 1997).

LI perseveration was reversed in Grin1^{D481N} mutants 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 (Lipina et al. 2005). Since both D-serine and ALX-5407 are highly selective for the glycine binding site (Atkinson et al. 2001; Matsui et al. 1995), NMDAR activation is the likely explanation for their capacity to overcome the effects of the Grin1^{D481N} mutation. In contrast, the mechanism by which clozapine reverses persistent LI has been proposed to involve antagonism of 5-HT$_{2A}$ receptors (Gaisler-Solomon and Weiner 2003; Weiner 2003). It has also been theorized that clozapine may exert its beneficial effects in animal models and in the clinic by enhancing NMDAR function (Millan 2005).

Activity-dependent redistribution of NMDARs may account for the reason glycinergic treatments and clozapine normalize persistent LI by slightly augmenting NPE scores in this and other studies (Gaisler-Solomon et al. 2008; Lipina et al. 2005). Recently, it has been shown that NMDAR glycine site stimulation promotes the priming of these receptors for clathrin-dependent endocytosis that occurs after their activation (Nong et al. 2003). Consequently, the reversal of persistent LI by compounds promoting NMDAR function may involve receptor internalization, which perhaps weakens nonessential circuits. In addition, reduced NMDAR activity in hippocampal synapses was found to recruit additional NMDARs to the synapse by lateral diffusion from extrasynaptic sites (Tovar and Westbrook 2002), and elevations in NMDAR subunit expression were observed in Grin1^{D481N} mutant mice (Kew et al. 2000). Thus, a larger pool of (abnormally functioning) NMDARs may explain why LI perseveration in Grin1^{D481N} mutant and MK-801-treated mice partially involves an enhancement in associative learning, as demonstrated by lowered NPE scores (Gaisler-Solomon et al. 2008; Lipina et al. 2005).

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 (Brodkin et al. 2004; O'Tuathaigh et al. 2007). The social approach impairment in the mutants 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 Grin1^{D481N} mutant mice and with NMDAR antagonists have reported anxiolytic behaviors (Kew et al. 2000; Wiley et al. 1995). 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 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 (Boulay et al. 2004; Bruins et al. 2005; Ellenbroek and Cools 2000; Sams-Dodd 1996). 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 (Murphy et al. 2006).

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 (Hlinak and Krejci 1995). The specific effect of the 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.

The Grin1^{D481N} mice were examined in a cognitive visuospatial 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 (Mandillo et al. 2003; Roullet et al. 1996). In addition, 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 (Mumby et al. 2002; Sargolini et al. 1999).

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. Notably the mutants reacted to the change by reexploring both displaced and nondisplaced 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 nonspatial 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 nonspatial novelty (Mandillo et al. 2003; Roullet et al. 1996). 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 the Grin1^{D481N} 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. In addition, the Grin1^{D481N} 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.

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