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Mutant mice with reduced NMDA-NR1 glycine affinity or lack of D-amino acid oxidase function exhibit altered anxiety-like behaviors

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ABSTRACT

Several compounds that promote activation of the *N*-methyl-D-aspartate receptor (NMDAR) glycine site have been proposed as treatments for schizophrenia, but the impact of these putative antipsychotics on anxiety remains unclear. In this study, we employed genetic and pharmacological mouse models of altered NMDAR glycine site function to examine the effects of these proposed treatments in unconditioned tests of anxiety. In the elevated plus-maze, open field, and novel object test, homozygous $Grin1^{D481N}$ mutant mice that have a five-fold reduction in NMDAR glycine affinity demonstrated an anxiolytic-like phenotype. In contrast, D-serine, a direct activator of the NMDAR glycine site, and ALX-5407, a glycine transporter-1 (GlyT-1) inhibitor, enhanced anxiety-like behaviors in wild-type and $Grin1^{D481N}$ mutant animals. Homozygous $Dao1^{G181R}$ mutant mice that lack function of the D-serine catabolic enzyme, D-amino acid oxidase (DAO), displayed an elevation in anxiety. Deficient DAO activity also reversed the anxiolytic effects of diminished NMDAR function in mice carrying both the homozygous $Grin1^{D481N}$ and $Dao1^{G181R}$ mutation. Thus, a direct agonist of the NMDAR glycine site, a GlyT-1 inhibitor, and suppression of DAO function induced anxiogenic-like behaviors. Consequently, application of these treatments for amelioration of schizophrenic symptoms necessitates caution as an enhancement of comorbid anxiety disorders may result.

1. Introduction

The prevalence of anxiety syndromes among patients with schizophrenia has long been recognized, with up to 62% of patients suffering from at least one comorbid anxiety disorder (Braga et al., 2005; Ciapparelli et al., 2007; Huppert and Smith, 2005). Coexisting anxiety disorders severely impact the prognosis of schizophrenia, and an increasing number of studies have shown an improvement of anxiety symptoms in schizophrenics treated with anti-anxiety medications (Braga et al., 2005; Kahn et al., 1988; Reznik and Sirota, 2000). This underscores the importance of proper adjunctive therapy for comorbid anxiety illnesses and the necessity to identify anti-psychotic treatments that will not aggravate symptoms of anxiety.

The glutamate system has been implicated in the pathophysiology of schizophrenia and anxiety disorders (Javitt, 2004; Krystal et al., 1999). Glutamatergic neurotransmission is mediated by several receptor subtypes, including the *N*-methyl-D-aspartate receptor (NMDAR). In addition to membrane depolarization, activation of the NMDAR requires concomitant binding to the NR1 glycine/D-serine site and to the NR2 glutamate site (Clements and Westbrook, 1991; Matsui et al., 1995). Research has indicated that NMDAR hypofunction is involved in the biological mechanism of schizophrenia, and suggests that enhanced NMDAR activity may be therapeutic (Coyle, 2006; Javitt, 2004). Since glutamate is potentially neurotoxic to cells, the glycine/D-serine site has been investigated as a potential target for novel antipsychotics (Coyle, 2006). Indeed, several clinical trials have tested the efficacy of direct or indirect modulators of the NMDAR glycine site, and have found improvements of schizophrenic symptoms when administered in conjunction with conventional medications (Heresco-Levy et al., 2005; Lane et al., 2006; Tsai et al., 1998, 2004).

Among the compounds that demonstrated success in clinical trials was D-serine, a selective full agonist of the NMDAR glycine site with an equal or greater potency than glycine (Heresco-Levy et al., 2005; Matsui et al., 1995; Tsai et al., 1998). Glycine transporter-1 (GlyT-1) inhibitors, such as ALX-5407, potentiate NMDAR activity by preventing glycine reuptake and consequently augmenting the extracellular level of glycine (Kinney et al., 2003; Lim et al., 2004). Inhibition of glycine transport was found to ameliorate the symptoms of schizophrenia in both medicated and drug-naïve patients, and was also demonstrated to be superior to D-serine in the treatment of acutely ill schizophrenics (Lane et al., 2005, 2006, 2008; Tsai et al., 2004). Blockade of the enzyme responsible for D-serine oxidation, D-amino acid oxidase (DAO), has also been proposed for

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antipsychotic treatment (Almond et al., 2006). Genetic inactivation of DAO in mice was shown to normalize the schizophrenia-like effect of NMDAR antagonist administration (Almond et al., 2006; Hashimoto et al., 2005b).

In rodents and humans, antagonists of the NMDAR, including compounds acting at glycine site, have been shown to produce anxiolytic-like behaviors (Bergink et al., 2004; Kotlinska and Liljequist, 1998; Krystal et al., 1994). However, the psychotomimetic properties of NMDAR inhibitors have hampered the pharmaceutical development of these compounds (Javitt and Zukin, 1991; Krystal et al., 1994). Since NMDAR blockade reduces anxiety, but also augments schizophrenia-like symptoms, it may follow that NMDAR glycine site activators, which have shown therapeutic potential in the treatment of schizophrenia, have additional adverse affects on anxiety-like behaviors. In this study, potential antipsychotic therapies involving the NMDAR glycine site were investigated to determine their effect on anxiety-like behaviors in mice. The effects of the direct agonist D-serine, the GlyT-1 inhibitor ALX-5407, and genetic inactivation of DAO function were assessed in unconditioned tests of anxiety: the elevated plus-maze, open field, and novel object task. Behavioral responses for each putative treatment were examined in wild-type mice and in the less anxious *Grin1*^{D481N} mutant mouse line (Kew et al., 2000). The *Grin1*^{D481N} mutation (aspartate to asparagine substitution at position 481) is located in the NR1 glycine binding site and confers a five-fold decrease in NMDAR glycine/D-serine affinity in the homozygous state (Kew et al., 2000). Biochemical and electrophysiological studies have confirmed that the reduced NMDAR glycine site function in mutant mice can be normalized by exogenous glycine and D-serine application (Duffy et al., 2008; Kew et al., 2000). Importantly, the *Grin1*^{D481N} mutant animals have been demonstrated to be a genetic model relevant to the negative and cognitive symptoms of schizophrenia with behavioral abnormalities that include persistent latent inhibition and impairments in sociability, spatial recognition, learning, and memory (Duffy et al., 2008; Labrie et al., 2008). In mutant animals, the behavioral perturbations related to schizophrenia were normalized by D-serine and ALX-5407, further supporting the therapeutic potential of these compounds (Labrie et al., 2008).

2. Materials and methods

2.1. Animals

Grin1^{D481N} transgenic mice were generated as previously described (Kew et al., 2000), and were derived from founders generously provided by Dr. M. Pauly-Evers, Hoffman-La Roche Ltd. (Basel, Switzerland). Prior to experiments, mice were backcrossed 11 generations onto the C57BL/6J strain. *Dao1*^{G181R} mice were obtained from the laboratory that identified a spontaneous missense mutation (glycine to arginine at amino acid 181) in the DAO gene of the ddY strain that results in a complete lack of DAO activity (Konno and Yasumura, 1983; Sasaki et al., 1992). We transferred the *Dao1*^{G181R} mutation onto a C57BL/6J genetic background, employing a marker-assisted speed congenic strategy. After six generations of backcrossing, the resultant *Dao1*$^{+/G181R}$ mice contained >99% of the C57BL/6J genome. Heterozygous *Dao1*^{G181R} and *Grin1*^{D481N} mice were crossed to produce *Dao1*$^{+/G181R}$; *Grin1*$^{+/D481N}$ mice. All animals used in this study were bred from heterozygous intercrosses (*Grin1*$^{+/D481N}$, *Dao1*$^{+/G181R}$, and *Dao1*$^{+/G181R}$; *Grin1*$^{+/D481N}$) in the animal colony at Mount Sinai Hospital, Toronto, Canada. Mice carrying the *Grin1*^{D481N} mutation were genotyped using a PCR-amplicon restriction endonuclease protocol (Duffy et al., 2008). Animals with the *Dao1*^{G181R} mutation were genotyped similarly using a primer pair (5′-TGATGTACGAAGCTGGAGGACA-3′ and 5′-TGTAGTGGCACCAGCTTT-3′) that amplified a 263 bp PCR product, which lacked an HpaII (Fermentas, Burlington, Canada) restriction site in homozygous mutants.

Mice were housed in groups of 3–5 same-sex littermates with *ad libitum* sterile food (Purina mouse chow) and water. The vivarium was maintained under a controlled temperature (21 °C±1 °C), humidity (50–60%) and 12 h light/dark cycle (lights on: 0700–1900). All animal procedures were approved by the Animal Management Committee of Mount Sinai Hospital and strictly followed the requirements of the Province of Ontario Animals for Research Act 1971 and the Canadian Council on Animal Care.

2.2. Behavioral studies

Behavioral testing was done between 0900 and 1300 hours on mice that were 8–12 weeks of age. Animals were randomized with regard to day and drug treatment, and sex-balanced, except where stated. Experiments were videotaped and scored by an observer blind to the genotypes. Experimentally naïve mice were assessed in the following order: elevated plus-maze, open field, and novel object test, with at least 1 week between tests, except in the pharmacological study where naïve mice were used for each task (Fig. 2). Prior to experiments, mice were left undisturbed in the room for at least 30 min to allow for acclimatization. The testing equipment was cleaned with 70% ethanol between each subject. Data collection was performed and analyzed using The Observer 5.0 (Noldus Information Technology, Wageningen, Netherlands).

2.2.1. Elevated plus-maze

The elevated plus-maze test was conducted as previously described (Soleimani et al., 2008; Young et al., 2008). 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. 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 ([open arm time/total time on apparatus]*100) and in % open arm entries ([open arm entries/total arm entries]*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.

2.2.2. Open field

The open field test was conducted as previously described (Young et al., 2008). The automated activity cage consisted of a transparent Plexiglas arena (41×41×31 cm) equipped with infrared beams to detect horizontal and vertical movements (model 7420/7430; Ugo Basile, Comerio, Italy). The mouse was placed at the center of the arena (68 lx) and allowed to explore the apparatus for 5 min. The time spent in central area (31×31 cm), in the periphery, freezing, and grooming was recorded (the sum of these measures=total time in apparatus). Horizontal and vertical activity (beam breaks) was also measured. Anxiolytic effects were indicated by an increase in % center time ([center time/total time in apparatus]*100), while anxiogenic effects were noted as a decrease in this measure. Horizontal and vertical activity was used to evaluate general motor activity.

2.2.3. Novel object

The novel object task was adapted from previously described studies (Avgustinovich et al., 2000; Heisler et al., 1998). Mice were individually housed for 1 week prior to the procedure. On the test day, a white plastic cylinder (6.2 cm height, 5.1 cm diameter) was carefully placed at the center of the home cage. The time spent exploring the object, the number of object contacts, and the duration of locomotor activity were recorded for 5 min. A mouse was considered to be

investigating the object if its snout was in contact with the object. Anxiolytic effects were observed if the % time exploring object ([object exploration time/total time]*100), and number of object contacts were increased, while a decrease in these measures indicated anxiogenic effects. Locomotion and general exploratory activity was assessed by the % time of locomotor activity ([locomotion time/total time]*100).

2.3. 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, pH adjusted to ~6 using 1 N NaOH. D-serine (600 mg/kg) was injected subcutaneously and ALX-5407 (1 mg/kg) was injected intraperitoneally. Drugs were administered at volume of 10 ml/kg with intervals of 20 min for D-serine and 120 min for ALX-5407 before testing. The doses of D-serine and ALX-5407 were chosen based on pilot dose response experiments and previous work with *Grin1*^{D481N} mice and C57BL/6J mice (Duffy et al., 2008; Labrie et al., 2008; Lipina et al., 2005).

2.4. Statistical analysis

Statistical analyses were conducted using Statistica (Statsoft Inc., Tulsa, OK, USA). Behavioral data were analyzed using ANOVA with the appropriate between-subjects factors. Significant main effects or interactions were followed by Fisher's least significant difference (LSD) post hoc comparisons. Statistical significance was set at $p<0.05$.

3. Results

3.1. *Grin1*^{D481N} mutant mice display a decrease in anxiety-like behaviors

Anxiety in *Grin1*^{D481N} mice was assessed in the elevated plus-maze, open field, and novel object test. The elevated plus-maze test

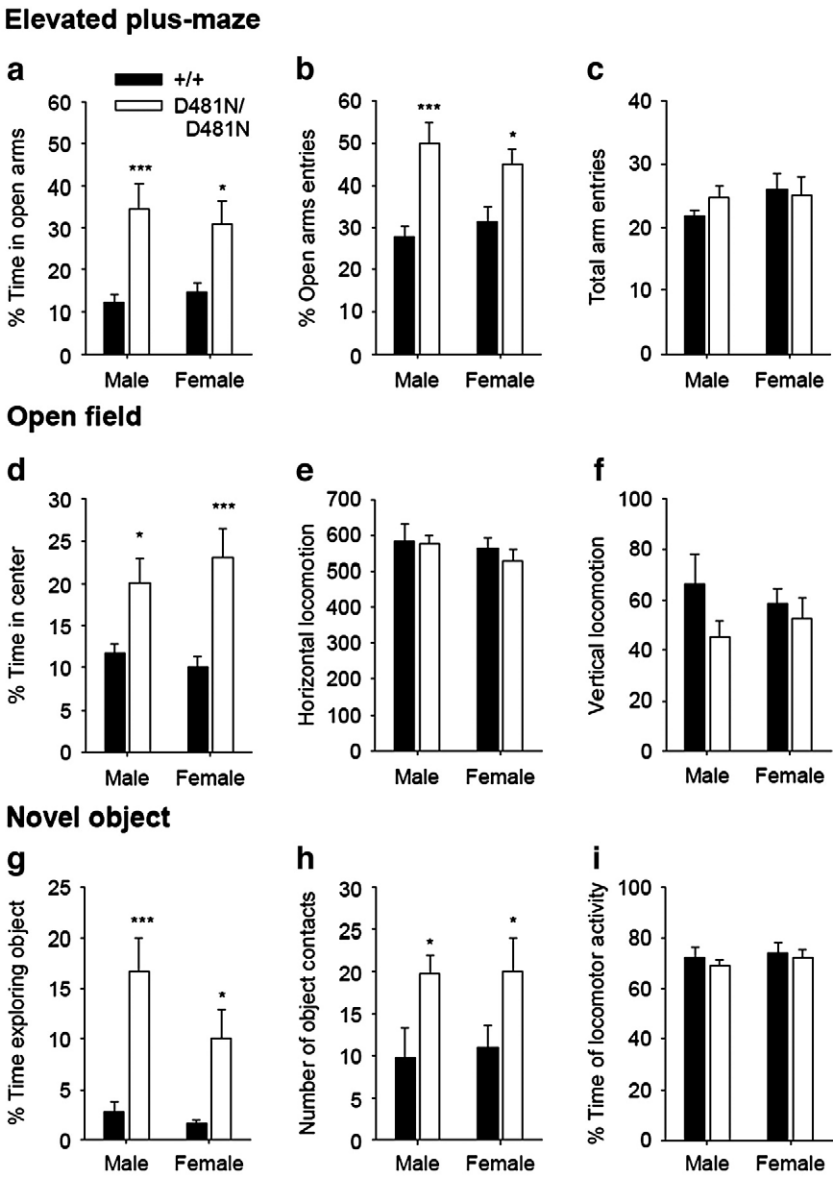

Fig. 1. Anxiolytic-like effects in mice with reduced NMDAR glycine affinity. In the elevated plus-maze, the amount of time spent in the open arms (a), the number of open arm entries (b), and the total number of arm entries (c) were measured in wild-type (+/+) and *Grin1*^{D481N} mutant (D481N/D481N) male and female animals (n=8–13/group). The time spent in the central area (d), and the horizontal (e) and vertical locomotion (f) were assessed in the open field test (n=11–12/group). Response to a novel object in a familiar environment was evaluated by examining the object exploration time (g), the frequency of object contacts (h), and the duration of locomotor activity (i) (n=8–12/group). * $p<0.05$, *** $p<0.001$ — compared to wild-type mice of the same sex.

exploits the conflict between the tendency of mice to investigate a novel environment and their tendency to avoid brightly lit, elevated, open areas (Crawley, 2000). In this test, homozygous mutant mice spent more time (main effect of genotype: $F(1,35)=21.65$, $p<0.001$) and made more entries into the open arms (main effect of genotype: $F(1,35)=23.43$, $p<0.001$) than wild-type animals (Fig. 1a and b). Post hoc analysis revealed that both male and female mutant mice displayed a greater amount of time ($p<0.05$) and entries into the open arms ($p<0.05$) than wild-type mice of the same sex. General locomotor activity did not differ between wild-type and mutant animals, as no differences were found in the total number of arm entries (Fig. 1c).

The open field is another measure of anxiety dependent on the natural aversion of mice for a brightly lit central area (Crawley, 2000). In contrast to wild-type mice, *Grin1*^{D481N} mutant animals spent significantly more time in the center of the open field (main effect of genotype: $F(1,42)=22.03$, $p<0.001$) (Fig. 1d). Within sex post hoc comparisons showed that male and female mutants had a greater % center time ($p<0.05$) than their wild-type counterparts. No differences were determined in horizontal and vertical locomotion (Fig. 1e and f) or in time spent freezing and grooming (data not shown).

State anxiety is a transient emotional response related to exposure to threatening stimulus, whereas trait anxiety is an enduring feature determining propensity for anxiety (Belzung and Berton, 1997). The elevated plus-maze and the open field tests have been described as measurements of state anxiety, whereas the novel object task in a familiar environment is proposed to assess trait anxiety (Avgustinovich et al., 2000; Belzung and Berton, 1997). In the novel object test, *Grin1*^{D481N} mutant mice spent substantially more time exploring the novel object (main effect of genotype: $F(1,33)=17.25$, $p<0.001$) and contacted the object more frequently (main effect of genotype: $F(1,33)=9.50$, $p<0.01$) than wild-type animals (Fig. 1g and h). Post hoc comparisons indicated that male and female mutant mice had a greater object exploration time ($p<0.05$) and number of object contacts ($p<0.05$) than wild-type mice of the same sex. A similar duration of locomotor activity was observed (Fig. 1i).

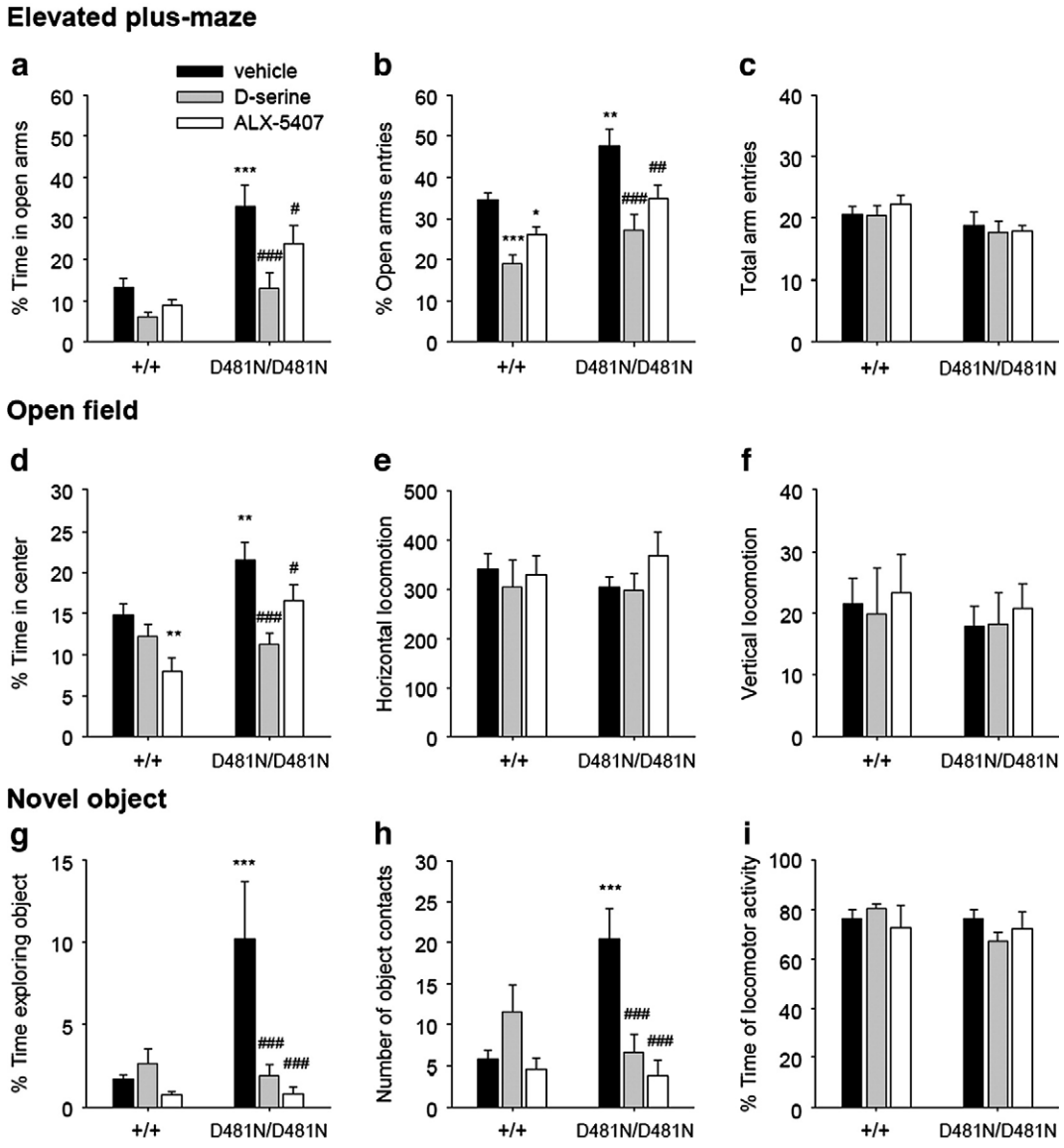

Fig. 2. The effects of D-serine or ALX-5407 treatment on anxiety-like behaviors in mice. The % time spent in the open arms of the elevated plus-maze (a), the number of open arm entries (b), and the total number of arm entries (c) were measured in wild-type (+/+) and *Grin1*^{D481N} mutant mice (D481N/D481N) given vehicle, D-serine (600 mg/kg), or ALX-5407 (1 mg/kg) ($n=16$–22/group). In the open field, the % center time (d), and the horizontal (e) and vertical activity (f) were evaluated ($n=15$–23/group). Object exploration time (g), frequency of object contacts (h), and % time of locomotion (i) were examined in the novel object task ($n=7$–9/group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ — compared to wild-type mice; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ — compared to mutant mice.

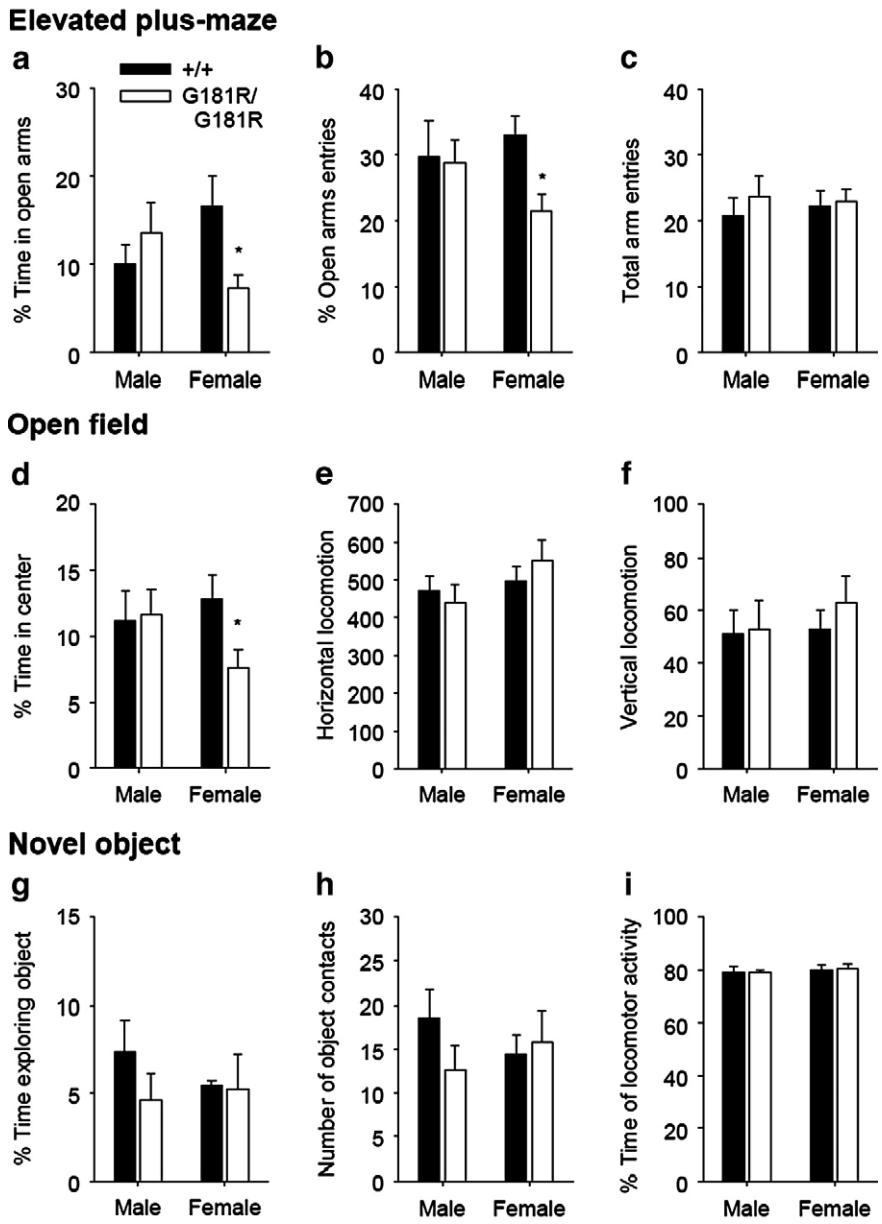

Fig. 3. Anxiety-like behaviors in mice carrying the *Dao1*^{G181R} mutation on the C57BL/6J strain. In the elevated plus-maze, the amount of time (a), the frequency of entries (b), and the total arm entries (c) were examined in wild-type (+/+) and *Dao1*^{G181R} mutant (G181R/G181R) male and female animals (n=11–14/group). The % time in the central area of the open field (d), and the horizontal (e) and vertical locomotion (f) were assessed (n=11–13/group). Response to a novel object was measured by evaluating the time spent exploring the object (g), the number of object contacts (h), and locomotor duration (i) (n=7–12/group). * $p<0.05$ — compared to wild-type mice of the same sex.

3.2. D-serine and ALX-5407 augment anxiety-like behaviors in wild-type and Grin1^{D481N} *mutant mice*

Compounds that promote NMDA-NR1 glycine site function, D-serine and ALX-5407, were administered to wild-type (*Grin1*$^{+/+}$) and *Grin1*^{D481N} mutant mice. The *Grin1*^{D481N} mutant animals allowed for the assessment of these compounds in a genetic mouse model relevant to schizophrenia (Labrie et al., 2008) and in animals displaying an anxiolytic phenotype. Since no sex differences were determined, male and female data were combined.

In the elevated plus-maze, D-serine and ALX-5407 enhanced anxiety-like behaviors in both wild-type and mutant mice. Analysis of the % time and entries into the open arms of the elevated plus-maze (Fig. 2a and b) revealed a significant main effect of genotype (% time: $F(1,107)=28.51$, $p<0.001$; % entries: $F(1,107)=17.50$, $p<0.001$) and drug treatment (% time: $F(2,107)=8.81$, $p<0.001$; % entries: $F(2,107)=18.54$, $p<0.001$). Post hoc comparisons showed that vehicle-treated mutants spent more time in the open arms ($p<0.001$) and entered the open arms more frequently ($p<0.01$) than vehicle-treated wild-types, and that this could be reversed by administration of D-serine ($p<0.001$) or ALX-5407 ($p<0.05$). Fewer open arm entries were also observed in wild-type mice given D-serine ($p<0.001$) or ALX-5407 ($p<0.05$) compared to vehicle-treated wild-type mice. D-serine and ALX-5407 did not affect overall motor activity, as the total number of arm entries did not differ between any group (Fig. 2c).

Similarly, an enhancement of anxiety-like behaviors following D-serine or ALX-5407 was also noted in the open field test. There were significant main effects of genotype, drug treatment, and genotype×drug treatment interaction in the % time spent in the center area (genotype: $F(1,108)=11.82$, $p<0.001$; drug treatment: $F(2,108)=9.61$, $p<0.001$; interaction: $F(2,108)=4.29$, $p<0.05$) (Fig. 2d). Post hoc analysis demonstrated that vehicle-treated mutants spent more

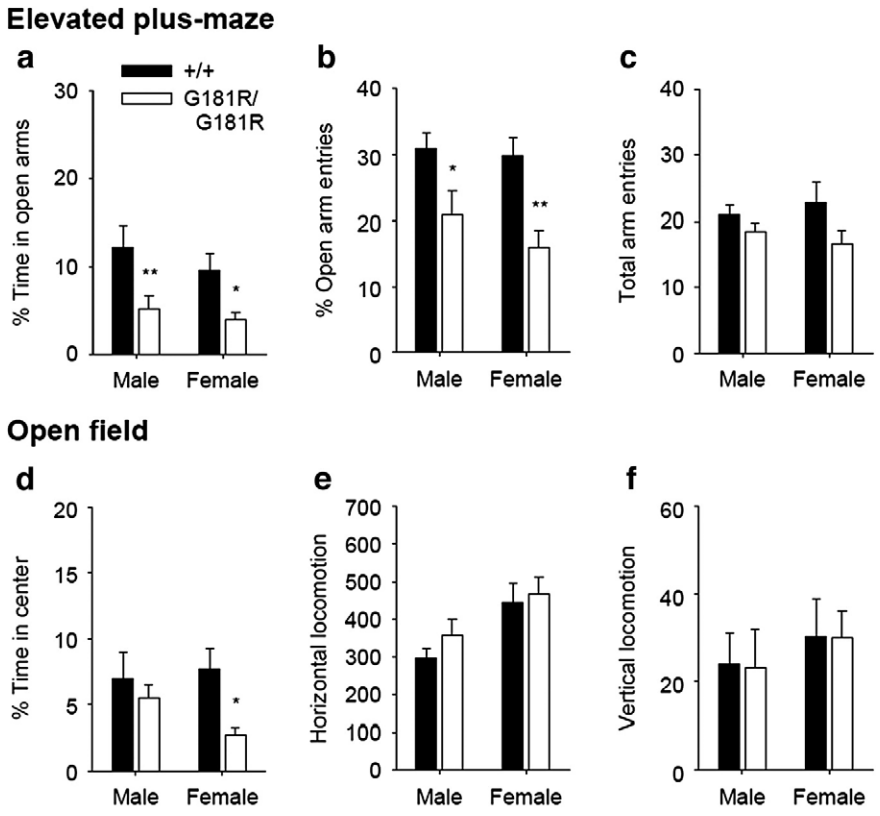

Fig. 4. Anxiety-like behaviors in mice with the *Dao1*^{G181R} mutation on the ddY strain. In the elevated plus-maze, the % time spent in the open arms (a), the number of open arm entries (b), and the total arm entries (c) were measured in wild-type (+/+) and *Dao1*^{G181R} mutant (G181R/G181R) male and female animals (n=8/group). The amount of time in the center of the open field (d), and the horizontal (e) and vertical locomotion (f) were also evaluated (n=10–13/group). * $p<0.05$, ** $p<0.01$ — compared to wild-type mice of the same sex.

time in the center ($p<0.01$) than vehicle-treated wild-types, and that this measure was reduced in mutants given D-serine ($p<0.001$) or ALX-5407 ($p<0.05$). Also, wild-type animals administered ALX-5407 demonstrated less time in the center ($p<0.01$) compared to vehicle-treated wild-type animals. Horizontal and vertical locomotion did not differ (Fig. 2e and f), and a similar duration of freezing and grooming was observed (data not shown).

D-serine and ALX-5407 reversed the anxiolytic effects of reduced NMDAR glycine site occupancy in the novel object task. Analysis of the % time spent exploring the object and number of object contacts (Fig. 2g and h) indicated a main effect of genotype (% time: $F(1,42)=4.53$, $p<0.05$), drug treatment (% time: $F(2,42)=6.24$, $p<0.01$; contacts: $F(2,42)=6.66$, $p<0.01$), and genotype×drug treatment interaction (% time: $F(2,42)=5.73$, $p<0.01$; contacts: $F(2,42)=8.92$, $p<0.001$). The object exploration time and contacts of mutant mice given D-serine or ALX-5407 was similar to vehicle-treated wild-types, and differed significantly from vehicle-treated mutants ($p<0.001$). Additionally, the amount of time spent and contacts with the object did not differ between wild-type animals given D-serine (contacts: $p=0.11$) or ALX-5407 compared to vehicle-treated wild-types. No differences in the duration of locomotor activity were detected (Fig. 2i).

3.3. *Dao1*^{G181R} mutant mice have an enhancement in anxiety-related behaviors

Mice with a complete lack of DAO function, the enzyme responsible for D-serine catabolism (Konno and Yasumura, 1983; Sasaki et al., 1992), were assessed in animal models of anxiety. In *Dao1*^{G181R} mice with a C57BL/6J background, anxiogenic behaviors were specifically increased in female mutant mice. A significant genotype×sex interaction was determined in the % time spent in the open arms of the elevated plus-maze ($F(1,45)=5.20$, $p<0.05$). Further analysis revealed that female *Dao1*^{G181R} mutants, but not male mutants, spent significantly less time ($p<0.05$) and made fewer entries into the open arms ($p<0.05$) compared to wild-type mice of the same sex (Fig. 3a and b). No differences were found in the total number of arm entries (Fig. 3c), suggesting that locomotor activity remained unaffected. Likewise, *Dao1*^{G181R} mutant females, but not males, displayed greater anxiety in the open field. In contrast to female wild-type mice, the % time spent in the center area of open field ($p<0.05$) was significantly reduced in *Dao1*^{G181R} mutant females (Fig. 3d). There were no differences in horizontal and vertical locomotor activity (Fig. 3e and f) or in the duration of freezing and grooming (data not shown). Additionally, the response to a novel object was similar in wild-type and mutant animals (Fig. 3g, h and i).

To confirm the effects of the *Dao1*^{G181R} mutation on anxiety in mice, subjects of the original ddY strain were tested in the tasks that had previously shown a behavioral disturbance in *Dao1*^{G181R} mice on the C57BL/6J strain. In accordance with our findings, increased anxiety-like behaviors in the elevated plus-maze and open field were demonstrated in *Dao1*^{G181R} mutant mice on the ddY strain (ddy-*Dao1*^{G181R}), particularly in female animals. In the elevated plus-maze, ddy-*Dao1*^{G181R} mutant mice displayed a reduction in the open arms time (main effect of genotype: $F(1,28)=13.33$, $p<0.01$) and frequency of open arms entries (main effect of genotype: $F(1,28)=18.04$, $p<0.001$) in comparison to wild-type animals (Fig. 4a and b). Within sex analysis indicated that both male and female mutants had a decrease in % time ($p<0.05$) and entries into the open arms ($p<0.05$) compared to their wild-type counterparts. Also, the total number of arm entries was not affected, suggesting normal locomotor activity (Fig. 4c). When ddy-*Dao1*^{G181R} mice were assessed in the open field, a main effect of genotype was found in the time spent in the central area ($F(1,41)=5.27$, $p<0.05$), since female mutant animals displayed a reduction in this measure compared to wild-type females ($p<0.05$)

(Fig. 4d). Horizontal and vertical locomotion (Fig. 4e and f) and the time spent freezing and grooming did not differ (data not shown).

3.4. The $Dao1^{G181R}$ mutation eliminates the anxiolytic behavioral effects of reduced NMDAR glycine affinity

Mice carrying both the $Dao1^{G181R}$ and $Grin1^{D481N}$ mutation were evaluated to determine whether the $Dao1^{G181R}$ mutation could genetically enhance the propensity for anxiety in subjects that are less anxious. Only male mice were used, since male $Dao1^{G181R}$ mutants on the C57BL/6J strain do not express elevated anxiety-like behaviors compared to wild-type males.

In the elevated plus-maze, the $Dao1^{+/+}$; $Grin1^{D481N/D481N}$ (+/$Grin1^{D481N}$) mice demonstrated a reduced aversion for the open arms that was completely abolished in the $Dao1^{G181R/G181R}$; $Grin1^{D481N/D481N}$ (double mutant) mice. A significant main effect of genotype was found in the % time spent in the open arms ($F(3,36)=11.08$, $p<0.001$) and the frequency of open arm entries ($F(3,36)=5.03$, $p<0.01$) (Fig. 5a and b). Post hoc comparisons revealed that +/$Grin1^{D481N}$ mice had a greater amount of % time ($p<0.001$) and entries ($p<0.01$) into the open arms compared to $Dao1^{+/+}$; $Grin1^{+/+}$ (wild-type) mice. Double mutant mice on the other hand displayed a similar amount of time and entries into the open arms as wild-type animals, and were significantly different from +/$Grin1^{D481N}$ mice ($p<0.05$). No significant differences were found with regard to the total number of arm entries (Fig. 5c).

In the open field, the $Dao1^{G181R}$ mutation again reversed the decreased anxiety-like behaviors resulting from diminished NMDAR function. There was a significant effect of genotype in the % time spent in the center of the open field ($F(3,34)=4.94$, $p<0.01$) (Fig. 5d). Post hoc analysis demonstrated that the elevated center time of +/$Grin1^{D481N}$

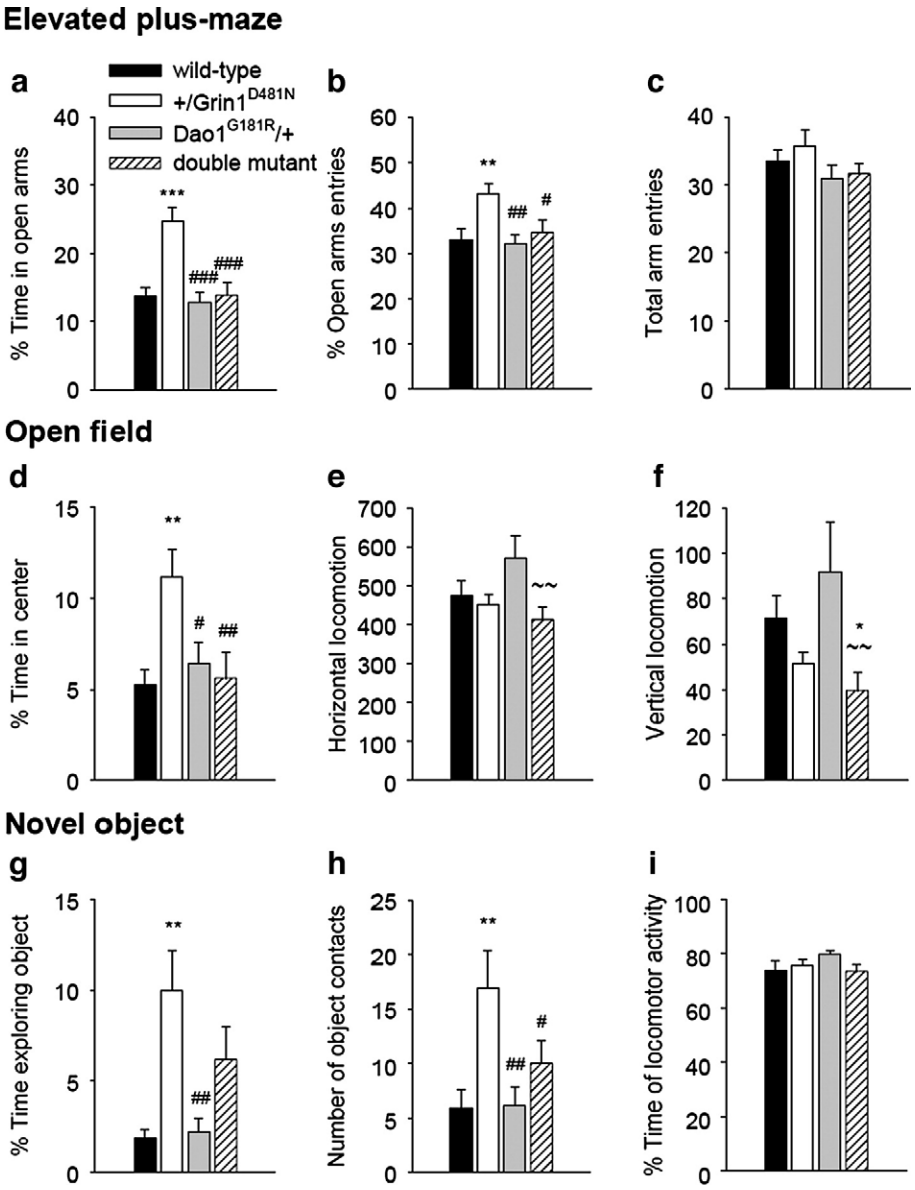

Fig. 5. The effect of a lack of DAO activity on the anxiolytic-like behaviors of mice with decreased NMDAR function. The % time spent in the open arms of the elevated plus-maze (a), the number of open arm entries (b), and the total number of arm entries (c) were measured in male $Dao1^{+/+}$; $Grin1^{+/+}$ (wild-type), $Dao1^{+/+}$; $Grin1^{D481N/D481N}$ (+/Grin1^{D481N}), $Dao1^{G181R/G181R}$; $Grin1^{+/+}$ (Dao1^{G181R}/+), and $Dao1^{G181R/G181R}$; $Grin1^{D481N/D481N}$ (double mutant) mice ($n=8$–13/genotype). The time spent in the center of the open field (d), and the horizontal (e) and vertical activity (f) were assessed ($n=11$–13/genotype). In the novel object task, the object exploration time (g), the number of object contacts (h), and the % time of locomotor activity (i) were examined ($n=9$–13/genotype). Animals in all tests carried mutations in the homozygous state, including the double mutant mice that had both the homozygous $Grin1^{D481N}$ and $Dao1^{G181R}$ mutation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ — compared to wild-type mice; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ — compared to +/Grin1^{D481N} mice; ~~ $p<0.01$ — compared to Dao1^{G181R}/+ mice.

mice compared to wild-type mice ($p<0.01$), was not present in double mutant animals. For horizontal locomotion (Fig. 5e), a significant effect of genotype ($F(3,34)=2.91$, $p<0.05$) was determined, due to *Dao1*$^{G181R/G181R}$; *Grin1*$^{+/+}$ (*Dao1*^{G181R}/+) mutants displaying greater locomotion than double mutants ($p<0.01$). A significant effect of genotype was also found in vertical locomotion ($F(3,34)=4.03$, $p<0.05$; Fig. 5f), as a result of the double mutants demonstrating less vertical activity than *Dao1*^{G181R}/+ mutant ($p<0.01$) and wild-type mice ($p<0.05$). The duration of freezing and grooming did not differ (data not shown).

During the novel object test, a lack of DAO activity partially attenuated the anxiolytic-like effects of decreased NMDAR function. A main effect of genotype was determined in the time spent exploring the object ($F(3,38)=5.72$, $p<0.01$) and the frequency of object contacts ($F(3,38)=4.41$, $p<0.01$) (Fig. 5g and h). Further analysis revealed that greater time ($p<0.01$) and contacts with the object ($p<0.01$) was demonstrated in+/*Grin1*^{D481N} mutant mice compared to wild-type mice, and that the number of object contacts was normalized in double mutant animals ($p<0.05$). Locomotor activity was similar between all genotypes (Fig. 5i).

4. Discussion

The direct NMDAR agonist D-serine, the GlyT-1 inhibitor ALX-5407, and genetic inactivation of DAO were shown to have prominent anxiogenic-like effects in mice in multiple tests of anxiety. These effects were not related to unspecific disruptions in motor function, as normal locomotor activity was observed in the elevated plus-maze, open field, and novel object task. The anxiogenic profile associated with these NMDAR modulators was further supported by their ability to attenuate diminished anxiety in *Grin1*^{D481N} mutant mice, which are also a genetic mouse model relevant to schizophrenia (Labrie et al., 2008). Though the NMDAR glycine site was stimulated using different approaches and in three distinct behavioral tasks, there was a substantial phenotypic consistency. Thus, treatments capable of activating the NMDAR glycine site produce a highly robust enhancement in anxiety-like behaviors in mice. These data indicate that the application of NMDAR glycine site modulators for the treatment of schizophrenia may have adverse effects on comorbid anxiety disorders.

The glutamatergic system is thought to have an important role in the pathogenesis of anxiety. Animal tests involving fear and stress produce an increase in NMDA-NR1 subunit expression in the ventral tegmental area (Fitzgerald et al., 1996), an augmentation of glutamate release in brain areas, such as the hippocampus, prefrontal cortex, and striatum (Bagley and Moghaddam, 1997; Ho et al., 2000), and alterations in the function or binding of the NMDAR and glycine site (Nowak et al., 1995; Yoneda et al., 1994). Stress-induced glutamatergic activation mediated by NMDARs produces excitotoxic effects in the hippocampus (Armanini et al., 1990; Krystal et al., 1999), and the neurotoxic effects of stress have been proposed to underlie the decrease in hippocampal volume associated with human posttraumatic stress disorder (Bremner et al., 1995). Conversely, anxiolytic-like effects have been observed following genetic inactivation of the NMDA-NR2A subunit in mice (Boyce-Rustay and Holmes, 2006). Pharmacological reduction of NMDAR function using competitive, non-competitive, and glycine site antagonists diminishes anxiety-like behaviors in preclinical rodent studies (Bergink et al., 2004; Kotlinska and Liljequist, 1998). Accordingly, the *Grin1*^{D481N} mutant mice, that have a five-fold reduction in NMDAR glycine site affinity, also demonstrated an anxiolytic phenotype. However, in contrast to the pharmacological studies, the *Grin1*^{D481N} mice are a model of chronic and developmentally decreased NMDAR function. Studies in rats and humans that have examined individual differences in anxiety levels indicate that occupancy of the NMDAR glycine site and/or glutamatergic activity may be reduced in naturally less anxious subjects, compared to highly anxious subjects (Ho et al., 2005; Tsang et al., 2007). Thus, mice with a chronic and developmental reduction in NMDAR glycine affinity may be better suited to explore the biological basis of low anxiety than transient pharmacological inhibition.

The potential clinical utility of NMDAR antagonists for the treatment of anxiety disorders has been greatly limited by their propensity to elicit unfavorable psychotomimetic side effects (Javitt and Zukin, 1991; Krystal et al., 1994). In fact, NMDAR antagonists, such as phencyclidine (PCP), induce schizophrenia-like psychosis, negative symptoms, and cognitive impairments in healthy subjects, and exacerbate symptoms in schizophrenic individuals (Javitt and Zukin, 1991; Krystal et al., 1994). NMDAR hypofunction has been strongly implicated in the pathogenesis of schizophrenia and this has spurred the investigation and development of novel antipsychotics that target the NMDAR glycine site (Coyle, 2006; Javitt, 2004). Several clinical trials have demonstrated that direct NMDAR agonists, such as D-serine, and inhibitors of GlyT-1 improve the symptoms of schizophrenic patients; however, none of the clinical studies reported the effects of these proposed therapies on anxiety (Heresco-Levy et al., 2005; Lane et al., 2005, 2006, 2008; Tsai et al., 1998, 2004).

This study revealed that administration of D-serine or the Gly-T1 inhibitor ALX-5407 augments anxiety-like behaviors in wild-type and *Grin1*^{D481N} mutant mice. Previously, infusions of D-serine or glycine into the dorsal periaqueductal gray area of the rat brain were shown to increase anxiety-like phenotypes (Schmitt et al., 1995; Santos et al., 2006). Though these studies do not assess the effects of a global enhancement of cerebral D-serine or glycine, they do provide support for the anxiogenic properties of these agents. Additionally, the behavioral effects of D-serine and ALX-5407 were more pronounced in *Grin1*^{D481N} mutants than in wild-type animals in these and other experiments (Duffy et al., 2008; Labrie et al., 2008). This indicates that the enhancement of anxiety by these compounds is more effective under conditions of reduced NMDAR function. Since diminished levels of D-serine and NMDAR activation have been reported in schizophrenic patients (Hashimoto et al., 2005a; Pilowsky et al., 2006), our results suggest that these compounds may have prominent anxiogenic effects in this population.

ALX-5407 may be more effective in increasing anxiety than D-serine, since this compound enhanced anxiety in wild-type mice in multiple paradigms, whereas D-serine produced effects in wild-type mice only in the elevated plus-maze. This could reflect a greater potentiation of NMDAR activity by GlyT-1 inhibitors compared to exogenous D-serine administration, made possible by the efficacy of GlyT-1 to tightly regulate extracellular glycine concentrations in NMDAR-expressing synapses (Kinney et al., 2003; Lim et al., 2004). Alternatively, the effects of ALX-5407 may be related to the activation of other glycine sensitive receptors. Unlike D-serine, glycine does not bind exclusively to the NMDAR, but also acts on inhibitory glycine receptors, which are present in brain regions relevant to anxiety, such as the amygdala, and are capable of modulating anxiety in rodents (McCool and Chappell, 2007; Zhang and Kim, 2007). Though GlyT-1 is closely associated to the NMDAR, while GlyT-2 is colocalized with the glycine receptor (Kinney et al., 2003), it remains possible that glycine receptors are involved in the anxiogenic profile of ALX-5407.

In contrast to our results, some reports have indicated that GlyT-1 inhibitors do not affect anxiety in mice and in certain studies high doses decreased responses to stress (Depoortère et al., 2005; Harsing et al., 2003; Rorick-Kehn et al., 2005; Yee et al., 2006). Some of these prior reports used highly anxious mouse strains, such as BALB/c and DBA/2, in which further increases in anxiety and stress reactivity would be difficult to detect (Belzung and Berton, 1997; Moy et al., 2007). However, differential dosing regimes of GlyT-1 antagonists likely account for the behavioral discrepancies. In our study, we employed a low dose of ALX-5407 compared to previous reports. Lower concentrations of Gly-T1 inhibitors have been shown to enhance NMDAR currents and LTP, while higher concentrations reduce

NMDAR currents and fail to augment LTP, since these higher doses promote NMDAR internalization (Martina et al., 2004; Nong et al., 2003). Consequently, high doses of a GlyT-1 inhibitor may result in a reduction of NMDAR activity that in turn diminishes stress responses, whereas low doses facilitate NMDAR function and enhance anxiety. Additionally, higher doses of ALX-5407 and sustained elevations of glycine have been described to have greater spill-over onto inhibitory glycineA receptors (Perry et al., 2008). Rodents given a 10 mg/kg dose of ALX-5407 were demonstrated to have high glycine levels in caudal brain regions along with respiratory and motor impairments (Perry et al., 2008). Thus, the behavioral effects associated with high doses of GlyT-1 inhibitors may involve an increased modulation of glycineA receptors.

The *Dao1*^{G181R} mutant mice displayed an anxiogenic-like phenotype on both the C57BL/6J and ddY genetic backgrounds, and this study is the first to examine anxiety-like behaviors in mutant animals that lack DAO activity. DAO is responsible for the degradation of a range of D-amino acids, and elevated levels of D-amino acids, including significantly increased D-serine, have been shown in the serum and brains of *Dao1*^{G181R} mutant mice (Hashimoto et al., 1993; Konno and Yasumura, 1983). Though it is possible that the heightened anxiety demonstrated by *Dao1*^{G181R} mice was related to changes in D-amino acids other than D-serine, the *Dao1*^{G181R} mutation was able to genetically attenuate the effects of the reduced NMDAR glycine affinity in double mutant mice. This argues that the behavioral changes identified in the *Dao1*^{G181R} mice are attributable to their action at the NMDAR glycine site. Accordingly, these mice have previously been shown to have increased LTP, an NMDAR-dependent form of synaptic plasticity, and resistance to glycine site and non-competitive NMDAR antagonists (Almond et al., 2006; Hashimoto et al., 2005b; Maekawa et al., 2005). Also, the capacity of acute D-serine treatments to exert similar enhancements in anxiety suggests that the phenotype exhibited by *Dao1*^{G181R} mice may not be related to potential abnormalities due to the presence of altered D-serine levels during development.

Interestingly, female *Dao1*^{G181R} mutant mice, but not males, were found to have a consistent increase in anxiety in the elevated plus-maze and open field tasks. The ability of the *Dao1*^{G181R} mutation to modulate anxiety-like behaviors in males was revealed when combined with the *Grin1*^{D481N} mutation. This signifies that the *Dao1*^{G181R} mutation can enhance anxiety in both sexes, but in females the behavioral effects may be more pronounced. The increased anxiety observed in *Dao1*^{G181R} female mice may be due to an enhanced sensitivity to the effects of chronically elevated D-serine. The ovarian steroid hormone estrogen is known to stimulate NMDAR function in the brain (Gazzaley et al., 1996). Estrogen also promotes synaptogenesis and dendritic spine formation in many brain areas during development and adulthood, through the activation of NMDARs (Gould et al., 1990; Schwarz et al., 2008; Woolley and McEwen, 1994). Furthermore, estradiol increases NR1 levels in dendrites of the hippocampus, and augments NR2A and NR2B levels in the hypothalamus (Gazzaley et al., 1996; Gore, 2001). Consequently, the augmented dendritic density and upregulation of NMDAR function by estrogen may render the *Dao1*^{G181R} female brain more susceptible to effects of sustained D-serine.

In our study, locomotion was not altered by the *Dao1*^{G181R} mutation in two distinct strains of mice. Conversely, Almond et al. (2006) found hypolocomotive effects in ddY-*Dao1*^{G181R} mutant mice. This discrepancy may be the result of procedural and apparatus differences, as locomotor activity is a measure that has been reported to be sensitive to experimental variation (Crabbe et al., 1999). Additionally, Almond et al. (2006) employed mice that were housed with environmental enrichment, which has been shown to affect a range of behavioral outcomes, including locomotor activity (Amaral et al., 2008; Varty et al., 2000). Environmental enrichment stimulates NMDAR-dependent synaptic plasticity, alters NMDAR subunit expression, and can differentially affect behaviors in animals with perturbed NMDAR function (Artola et al., 2006; Bredy et al., 2004; Tang et al., 2001). Thus, it is conceivable that the previously reported change in locomotor activity in the ddY-*Dao1*^{G181R} mice is related to a differing response to environmental enrichment. Locomotor activity in mice can also be modulated by stressful environments (Mineur et al., 2006; Strekalova et al., 2004). In the experiment involving the double mutant animals (Fig. 5), locomotor behaviors were altered in the open field. In contrast to mice in our earlier experiments, mice in these tests were located in close proximity to the entrance of the vivarium, a location that has greater exposure to potentially stressful noise and visual stimuli (Sherwin, 2002). Furthermore, wild-type animals in the double mutant line demonstrated a higher basal anxiety compared to wild-type mice in earlier experiments. Though relative anxiety between genotypes remained consistent, the enhancement in basal anxiety supports the notion that mice in the double mutant experiment experienced greater stress. Therefore, certain environmental conditions are capable of producing differences in locomotor behavior.

Numerous genetic linkage studies have implicated the DAO gene and its activator, DAOA (G72/G30), in schizophrenia susceptibility (Chumakov et al., 2002; Corvin et al., 2007b; Hong et al., 2006; Liu et al., 2004; Yue et al., 2007). Interestingly, DAOA has also been associated with an increased risk of panic disorder in a German population sample (Schumacher et al., 2005), and is located within the chromosomal region, 13q32–q33, that was found to be the strongest linkage peak for panic disorder syndrome in a genome-wide scan involving 60 multiplex pedigrees (Hamilton et al., 2003). Furthermore, a risk allele for DAO, shown in four independent studies to be associated with schizophrenia, was also linked to greater symptoms of anxiety and guilt in Irish schizophrenic patients (Corvin et al., 2007a). Although these studies are preliminary, they indicate an overlap of certain proteins and neurotransmitter systems involved in the etiology of schizophrenia and anxiety disorders. Consequently, drugs targeting these common systems may influence symptoms in both disorders.

In conclusion, a direct agonist of the NMDAR glycine site, a GlyT-1 inhibitor, and the loss of DAO function produced anxiogenic-like effects in mice. Compounds promoting NMDAR glycine site activity, such as those examined in this study, have been proposed as novel treatments for schizophrenia. Considering the high prevalence of anxiety disorders and symptomatology in schizophrenic patients, novel therapies targeting the NMDAR glycine site may require extensive investigation to ensure that there is no induction or exacerbation of anxiety syndromes.

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