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Mice with Reduced NMDA Receptor Expression Display Behaviors Related to Schizophrenia

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Summary

N-methyl-D-aspartate receptors (NMDARs) represent a subclass of glutamate receptors that play a critical role in neuronal development and physiology. We report here the generation of mice expressing only 5% of normal levels of the essential NMDAR1 (NR1) subunit. Unlike NR1 null mice, these mice survive to adulthood and display behavioral abnormalities, including increased motor activity and stereotypy and deficits in social and sexual interactions. These behavioral alterations are similar to those observed in pharmacologically induced animal models of schizophrenia and can be ameliorated by treatment with haloperidol or clozapine, antipsychotic drugs that antagonize dopaminergic and serotonergic receptors. These findings support a model in which reduced NMDA receptor activity results in schizophrenic-like behavior and reveals how pharmacological manipulation of monoaminergic pathways can affect this phenotype.

Introduction

NMDA receptors, together with AMPA and kainate receptors, comprise the known ionotropic glutamate receptors (Nakanishi et al, 1998). NMDA receptors have generated considerable interest due to their unique pharmacological and electrophysiological properties and their role in synapse refinement, neuronal plasticity, and excitotoxicity (Nakanishi et al., 1998). These receptors require concurrent membrane depolarization and glutamate binding for activation. Under these conditions, Ca^{2+} influx through the NMDA receptor channel provides a second messenger system linking synaptic activity to long-term changes in synaptic efficacy (Nakanishi et al., 1998). Functional NMDA receptors are composed of a common NR1 subunit and one of four NR2 subunits (NR2A–NR2D) combined in an undetermined ratio to make the heteromeric receptor complex (Kutsuwada et al., 1992; Monyer et al., 1992). An inhibitory NR3 subunit may also be present in the receptor complex, particularly during development (Das et al., 1998).

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Mice lacking each of the known NMDA receptor subunits have been generated (Forrest et al., 1994; Li et al., 1994; Ikeda et al., 1995; Sakimura et al., 1995; Ebralidze et al., 1996; Kutsuwada et al., 1996; Das et al., 1998). Mice lacking NR1 or NR2B protein die perinatally (Forrest et al., 1994; Li et al., 1994; Kutsuwada et al., 1996). As a result, mice deficient in all NMDA receptors cannot be used to study diseases that have been hypothesized to be associated with NMDA receptor deficiency, such as schizophrenia (Javitt and Zukin, 1991; Coyle, 1996; Carlsson et al., 1997; Goff and Wine, 1997; Tamminga, 1998).

Schizophrenia is characterized by episodic positive symptoms such as delusions, hallucinations, paranoia, and psychosis and/or persistent negative symptoms such as flattened affect, impaired attention, social withdrawal, and cognitive impairments (Ban et al., 1984). A major explanatory hypothesis for the pathophysiology of schizophrenia is the dopamine hypothesis, which maintains that dysfunction of the dopamine neurotransmitter system underlies the behavioral abnormalities that accompany schizophrenia. This hypothesis is based on the observation that drugs effective in treating schizophrenia share the common feature of blocking dopamine receptors (Anden et al., 1970; Seeman et al., 1976). In many respects the behavior of patients with schizophrenia is consistent with elevated levels of dopaminergic neurotransmission, and some of the symptoms of schizophrenia can be reproduced by drugs such as amphetamine that increase dopaminergic tone (Griffith et al., 1972; Angrist et al., 1974). However, altered levels of dopamine or dopamine receptors have not generally been observed upon postmortem examination of the brains of schizophrenic patients (Knable et al., 1994; Lahti et al., 1996; Carlsson et al., 1997).

An alternate explanation for the etiology of schizophrenia is the glutamate dysfunction hypothesis. This hypothesis originated from the observation that phencyclidine (PCP) intoxication closely mimics schizophrenia (Luby et al., 1959). At serum levels that produce schizophrenic symptoms, PCP acts as a noncompetitive antagonist of NMDA receptors (Javitt and Zukin, 1991). Ketamine and MK-801, two additional noncompetitive antagonists of NMDA receptors, also produce schizophrenic symptoms (Ellison, 1995; Malhotra et al., 1997; Abi-Saab et al., 1998) and exacerbate symptoms in patients with schizophrenia (Javitt and Zukin, 1991). These findings led to the use of PCP- and MK-801-treated animals as models for schizophrenia (Carlsson and Carlsson, 1990; Corbett et al., 1993, 1995).

The glutamate dysfunction hypothesis is not inconsistent with an important role for dopamine in the pathogenesis of this disease or with the current therapeutic approach for schizophrenia, which relies on drugs that act through dopamine receptor blockade or the combined antagonism of dopamine and serotonin receptors (Meltzer, 1991). Glutamate and dopamine have been reported to exhibit reciprocal actions at subcortical

structures (Nieollon et al., 1983), and therefore dopamine receptor blockade may act to balance glutamatergic insufficiency (Carlsson et al., 1997).

We describe here the production of a genetically altered mouse line that expresses 5%–10% of the normal level of *Nr1*. While these mice are deficient in NMDA receptors, they express sufficient levels to allow survival to adulthood. *Nr1* mutant mice display behavioral abnormalities that have been related to schizophrenia, and these behaviors are ameliorated by the antipsychotic drugs haloperidol and clozapine. The greater efficacy of clozapine over haloperidol in the treatment of these mice and the observation of unchanged levels of dopaminergic transmission suggest that the abnormal behaviors are produced without major modifications of the dopaminergic system. These results provide evidence supporting the hypothesis that dysfunction of glutamatergic pathways, specifically those utilizing NMDA receptors, can lead to the pathologic changes that characterize schizophrenia.

Results

Generation of a Hypomorphic Allele of *Nr1*

A mouse line expressing reduced levels of the NR1 subunit of NMDA receptors was generated using homologous recombination in embryonic stem cells. A targeting vector was designed that, upon integration into the *Nr1* locus (now designated *Grin1*), resulted in the insertion of a neomycin resistance gene into intron 20 (Figure 1). ES cells carrying the targeted mutation were used to generate mice with an altered *Nr1* allele ($Nr1^{neo}$ +/−). These animals were intercrossed and used to examine the impact of the intronic insertion on *Nr1* expression and on the survival of $Nr1^{neo}$ −/− mice. No perinatal lethality was observed, and $Nr1^{neo}$ homozygous mice were identified at expected frequency at weaning. $Nr1^{neo}$ −/− mice were slightly smaller than littermates. Although the weight difference between the two groups persisted into adulthood, the magnitude of the difference diminished, and the majority of $Nr1^{neo}$ −/− mice reached weights within the normal range.

To examine the impact of the intronic insertion on *Nr1* expression, RNA was prepared from whole brain extracts of wild-type, $Nr1^{neo}$ +/−, and $Nr1^{neo}$ −/− mice and subjected to Northern blot analysis. *Nr1* message levels were reduced in the brains of $Nr1^{neo}$ −/− mice to 8.1% ± 1.3% of normal levels (Figure 1). Western blot analysis indicated that NR1 protein levels were 7.3% ± 1.4% of that seen in wild-type mice (Figure 1). Novel mRNA transcripts or proteins were not observed by Northern or Western blot analysis, indicating that the intronic insertion did not lead to the production of novel splice variants from this allele.

The *Nr1* mRNA produced in the brains of mutant mice was cloned by RT–PCR and sequenced to verify that alterations of the primary sequence were not introduced by the targeting construct or during the recombination event. The sequence of *Nr1* cDNA from the mutant mice did not differ from published sequences for this gene. Thus, the insertion of the *neo* gene into intronic *Nr1* sequences resulted in a severe reduction in expression of *Nr1* without introducing point mutations or generating

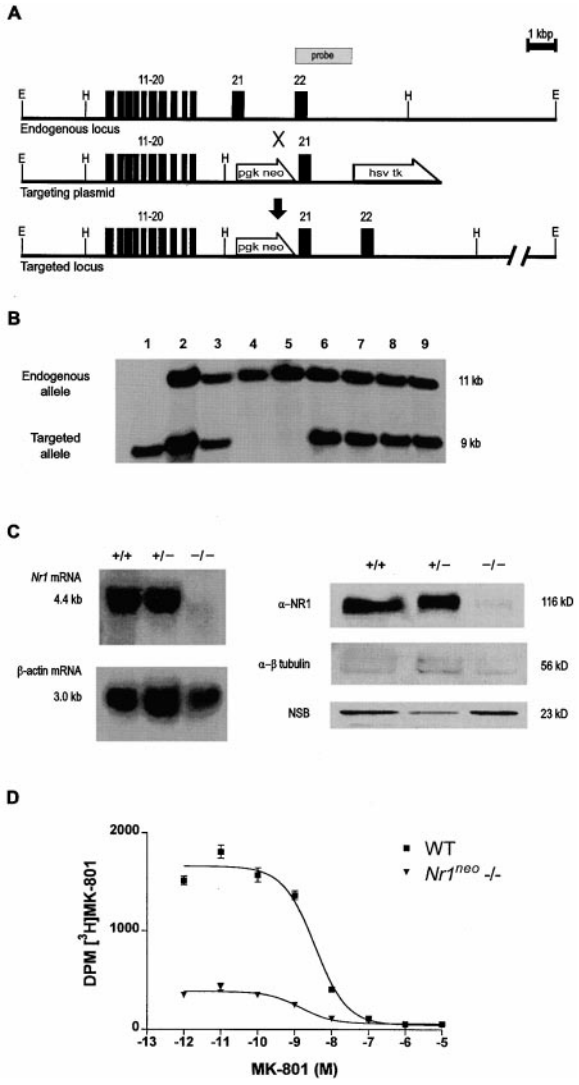

Figure 1. Targeted Insertion of *neo* into *Nr1* Intron 20 Results in Decreases in *Nr1* mRNA, Protein, and Functional NMDA Receptors

(A) Homologous recombination of the targeting plasmid with the endogenous *Nr1* locus results in insertion of the *neo* gene into intron 20 of *Nr1* and the introduction of a new HindIII restriction site.

(B) An 11 kb HindIII fragment corresponding to the endogenous NR1 locus and a 9 kb HindIII fragment from the mutant allele can be distinguished by Southern blot analysis of HindIII-digested DNA with the probe indicated. (H, HindIII; E, EcoRI).

(C) Northern (left panel) and Western (right panel) analysis of NR1 expression in total RNA and protein extracts prepared from brains of *Nr1* +/+, $Nr1^{neo}$ +/−, and $Nr1^{neo}$ −/− mice. Analysis of β-actin expression verifies that the amount and integrity of the RNA loaded in each lane are similar. NR1 protein levels are quantified by Western analysis, and equivalent loading is demonstrated using antibodies specific for β-tubulin and with the nonspecific binding (NSB) at 23 kDa seen using the antibody directed toward NR1.

(D) Radioligand binding of [^{3}H]MK-801 to membrane homogenates from the prefrontal cortex of *Nr1*+/+ and $Nr1^{neo}$ −/− mice. A competition binding curve was generated using 2 nM [^{3}H]MK-801 and increasing concentrations of MK-801 to calculate the IC_{50} and B_{max} of MK-801 binding. The IC_{50} for both the wild-type and mutant mice is 2 nM. The B_{max} is 0.12 pmol/mg for the $Nr1^{neo}$ −/− and 1.2 pmol/mg for wild-type mice (n = 3 for each genotype).

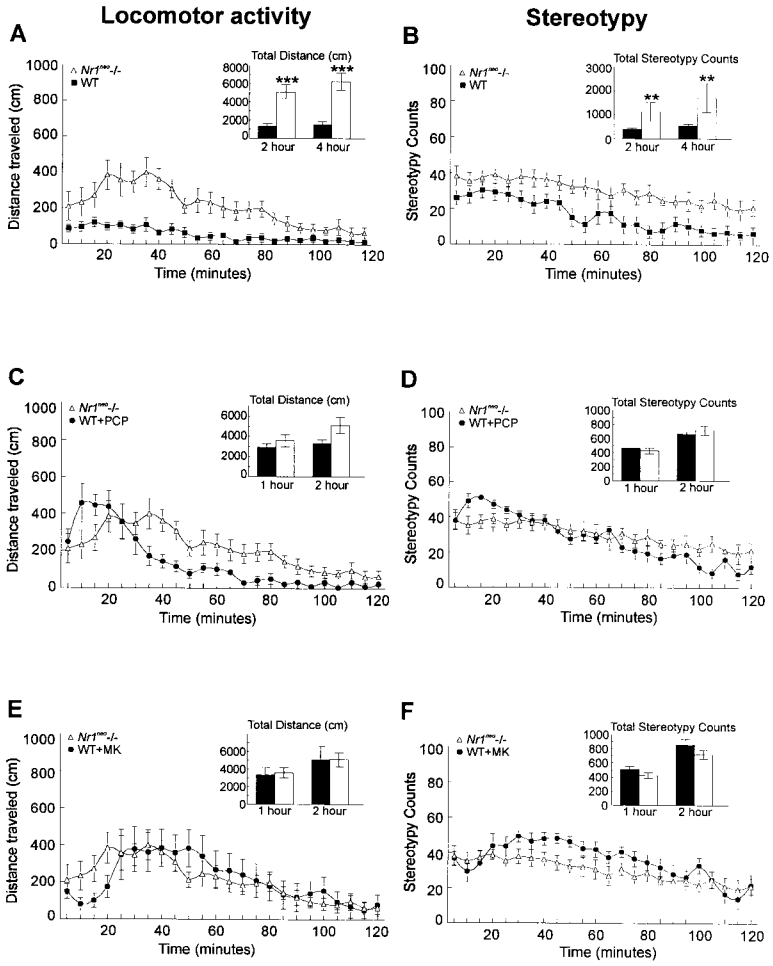

Figure 2. *Nr1*^{neo} −/− Animals Display Increases in Locomotion and Stereotypy Similar to Those Seen in Pharmacological Models of Schizophrenia

(A) Locomotor activity and (B) stereotypy of untreated *Nr1*^{neo} −/− mice (open bars, open triangles) and wild-type littermates (filled bars, filled circles). Two-way ANOVA, $p < 0.0001$, $F = 159.7$. (C) Locomotor activity and (D) stereotypy of untreated *Nr1*^{neo} −/− mice (open bars, open triangles) and wild-type littermates treated with 3 mg/kg PCP, i.p. (filled bars, filled circles). Two-way ANOVA analysis of locomotor, $p = 0.0912$. (E and F) Same as above, except that wild-type mice are treated with 0.2 mg/kg MK-801 i.p. Two-way ANOVA of locomotor activity, $p = 0.9521$. For (A)–(F), *** = $p < 0.0005$, ** = $p < 0.005$, two-tailed Student's t test. $n = 8$ for all groups.

novel transcripts or proteins. This indicated that the *Nr1*^{neo} mutation produces a hypomorphic allele.

Expression of functional NMDA receptor in the prefrontal cortex of both wild-type and mutant animals was determined by radioligand binding using the NMDA receptor antagonist, [^{3}H]MK-801. Analysis of competition binding curves generated with homogenates prepared from mutant and wild-type animals indicated that, as expected, the IC_{50} for both animals was identical (Figure 1). However, the calculated maximal binding of [^{3}H]MK-801 in the *Nr1*^{neo} −/− was only 10% (0.12 pmol/mg) of that of wild-type animals (1.2 pmol/mg).

Hyperlocomotion and Increased Stereotypic Behaviors in *Nr1*^{neo} −/− Mice

Animal models of schizophrenia induced by treatment with phencyclidine (PCP) or amphetamine display increases in both locomotion and stereotypic behaviors, and these behaviors have been correlated with the positive symptoms of schizophrenia (Corbett et al., 1995; Moghaddam and Adams, 1998). Motor activity was recorded over a 2–4 hr time period in a digital activity monitor. During the first 2 hr of monitored activity, *Nr1*^{neo} −/− mice behaved with increased ambulation relative to wild-type controls (3.9× greater, $p = 0.0001$) and displayed increased stereotypic movements (2.9× greater, $p = 0.005$) (Figure 2). The increased motor activity occurred during habituation to a new environment, since the motor activity of *Nr1*^{neo} −/− mice was reduced to that of wild-type mice after 4 hr (data not shown).

The increases in locomotion and stereotypy seen in *Nr1*^{neo} −/− mice were both qualitatively and quantitatively similar to that observed following administration of the NMDA receptor antagonists PCP or MK-801 (Figure 2). Wild-type mice injected with 3 mg/kg PCP or 0.2 mg/kg MK-801 demonstrated a pattern of motor activity similar to untreated *Nr1*^{neo} −/− mice, and no statistical differences were found between the two groups.

PCP and MK-801 Lack Effects in *Nr1*^{neo} −/− Mice

Nr1^{neo} −/− mice were also treated with PCP and MK-801 to address the question of whether these drugs elicit their motor stimulatory effects solely through their interaction with NMDA receptors. PCP has been shown to interact with other neurotransmitter systems, including the dopaminergic and serotonergic systems (Akunne et al., 1992; Rothman, 1994), and it has been proposed that these interactions are responsible for the motor stimulatory effects seen in rodents. If this were the case, it would be expected that the administration of PCP would lead to increases in motor activity beyond what is seen in untreated *Nr1*^{neo} −/− mice. However, *Nr1*^{neo} −/− mice did not display increases in locomotion or stereotypy when injected with doses of MK-801 or PCP selected to elicit maximal motor stimulation (Gleason and Shannon, 1997) (Figure 3). Both drugs were

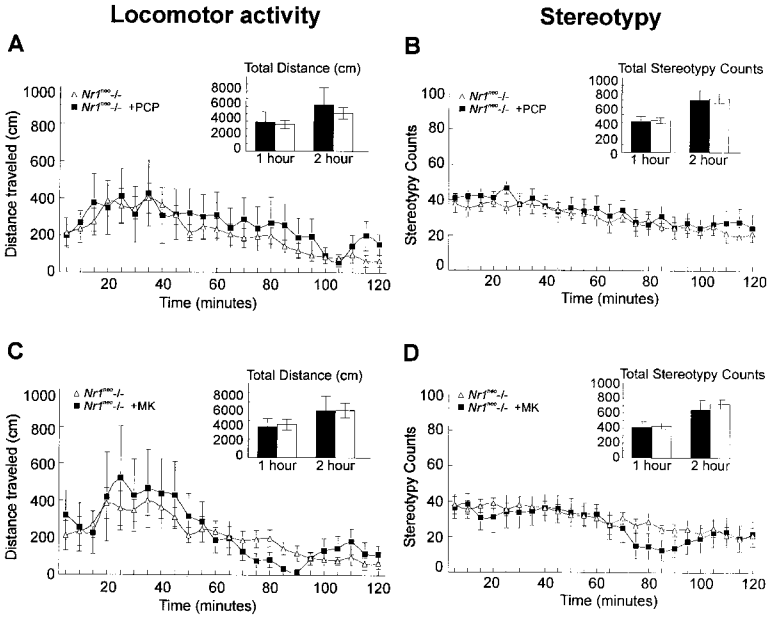

Figure 3. Lack of Motor Stimulatory Effects of PCP and MK-801 in *Nr1*^{neo} −/− Mice

(A) Locomotor activity and (B) stereotypy of untreated *Nr1*^{neo} −/− mice (open bars, open triangles) and *Nr1*^{neo} −/− mice treated with 3 mg/kg PCP, i.p. (filled bars, filled squares). (C and D) Same as above, except that *Nr1*^{neo} −/− mice are treated with 0.2 mg/kg MK-801, i.p. (filled bars, filled squares). Two-way ANOVA analysis of locomotor activity, $p = 0.4572$. $n = 8$ for all groups. p values calculated by a two-tailed Student's t test are greater than 0.5 for all comparisons of horizontal activity and stereotypic behavior.

ineffective as motor stimulants in *Nr1*^{neo} −/− mice, suggesting that at these doses their effects on behavior were mediated solely through the direct suppression of NMDA receptors.

Attenuation of Abnormal Motor Behaviors with Antipsychotics

The "typical" antipsychotic drug haloperidol and the "atypical" antipsychotic drug clozapine were tested for their ability to attenuate the hyperlocomotion and stereotypy of *Nr1*^{neo} −/− mice. Haloperidol, a potent D_2-dopamine receptor antagonist with relatively high specificity (Hyttel, 1978), reduced these behaviors in *Nr1*^{neo} −/− animals to wild-type levels of activity (Figure 4). Although this result suggests that D2 dopaminergic pathways are responsible for these behaviors, the same dose of haloperidol also resulted in substantial impairment of the locomotor activity of wild-type mice. This effect of haloperidol on control mice is not surprising given the therapeutic profile of this drug, which has marked extrapyramidal side effects (EPS) at its effective antipsychotic dose in humans (Hoffman and Donovan, 1995).

In contrast, clozapine and other atypical antipsychotics can suppress psychotic symptoms without EPS (Ereshefsky et al., 1989; Gerlach, 1991). Clozapine's pharmacological profile includes antagonism at both D_1- and D_2-like dopamine receptors and 5HT2 receptors and also interactions with α_1-adrenergic and H1 histamine receptors (Ereshefsky et al., 1989; Gingrich and Caron, 1993). Clozapine was effective at attenuating the abnormal motor behavior of *Nr1*^{neo} −/− mice at a dose that did not affect the motor behavior of wild-type littermates (Figure 4).

The efficacies of clozapine and haloperidol were further studied by examining the ability of either drug to attenuate motor activity over a range of doses (Figure 5). The ED_{50} of clozapine on the locomotor activity of *Nr1*^{neo} −/− mice was 0.08 mg/kg. Remarkably, this dose was 40× lower than the ED_{50} of clozapine for suppression of locomotion in wild-type mice (3.2 mg/kg). In contrast, the ED_{50} of haloperidol on the activity of *Nr1*^{neo} −/− mice was 0.29 mg/kg, double the ED_{50} of 0.09 mg/kg for wild-type activity.

Dopamine Release and Metabolism Are Unchanged in *Nr1*^{neo} −/− Mice

The neural pathways that mediate the increased motor activity seen upon blockade of the NMDA receptor have

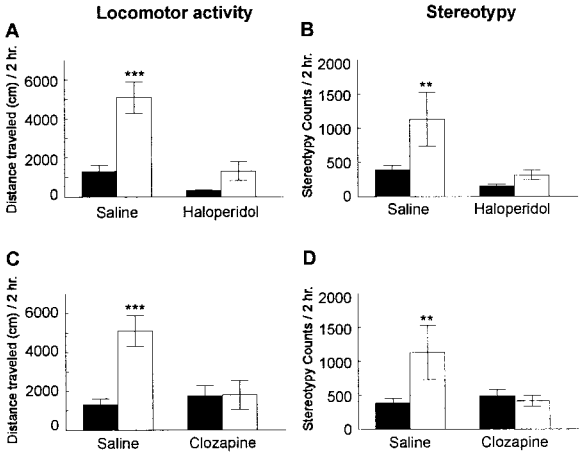

Figure 4. Administration of Haloperidol and Clozapine Attenuates Abnormal Behaviors of *Nr1*^{neo} −/− Mice

(A) Locomotor activity and (B) stereotypy of *Nr1*^{neo} −/− mice (open bars) and their wild-type littermates (filled bars) following administration of either saline or 0.5 mg/kg haloperidol, i.p. (C and D) Same as above, except that mice were treated with either saline or 0.5 mg/kg clozapine, i.p. For (A)–(D), *** = $p < 0.0005$, ** = $p < 0.005$, Student's two-tailed t test. $n = 8$ for all groups.

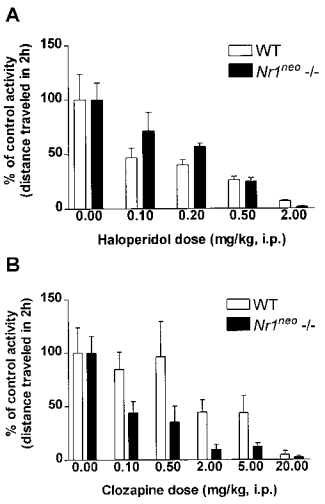

Figure 5. Clozapine Is More Effective Than Haloperidol in the Attenuation of Hyperlocomotion of *Nr1*^{neo} −/− Mice

(A) Dose dependence of haloperidol's effects on the motor activity of wild-type (filled bars) and *Nr1*^{neo} −/− mice (open bars), illustrated as the percentage of control activity. Estimated ED_{50} of effect of haloperidol on the locomotor activity is 0.09 mg/kg (95% confidence interval of 0.02–0.40) in wild-type mice and 0.29 mg/kg (95% confidence interval of 0.14–0.61) in *Nr1*^{neo} −/− mice.

(B) Animals were treated in a similar manner with clozapine. Estimated ED_{50} of effect of clozapine on the locomotor activity is 3.2 mg/kg (95% confidence interval of 0.35–30.5) in wild-type mice and 0.08 mg/kg (95% confidence interval of 0.01–0.37) in *Nr1*^{neo} −/− mice. For clozapine, two-way ANOVA analysis of dose response reveals a significant genotype x drug response ($p = 0.0087$). n = 6–8 for each dose.

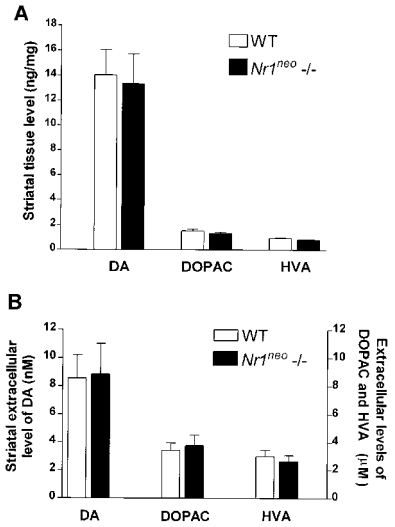

Figure 6. Parameters of Striatal Dopamine Neurotransmission in *Nr1*^{neo} −/− and Wild-Type Mice

(A) Tissue content of dopamine (DA) and its metabolites (DOPAC, HVA) in the striatum of *Nr1*^{neo} −/− mice (filled bars, n = 4) and wild-type mice (open bars, n = 4) measured by HPLC.

(B) Extracellular dopamine levels in the striatum of freely moving mice measured using quantitative "low perfusion rate microdialysis." Wild-type mice (open bars, n = 5), *Nr1*^{neo} −/− mice (filled bars, n = 4).

not been established. Because hyperactivity is commonly associated with increased dopaminergic tone, it has been postulated that NMDA receptor antagonists exert their effect by either directly or indirectly increasing dopaminergic tone (Imperato et al., 1990; Miller and Abercrombie, 1996). However, studies of dopamine release and metabolism following pharmacological NMDA receptor antagonism have generated conflicting results, and several groups have not observed alterations in dopamine transmission (Druhan et al., 1996; Pierce et al., 1997). To address this question, the extracellular concentration of dopamine release was measured in the striatum of freely moving *Nr1*^{neo} −/− and wild-type mice by quantitative in vivo microdialysis (Wang et al., 1997) (Figure 6). Levels of both dopamine and dopamine metabolites were unchanged in the striatum of *Nr1*^{neo} −/− mice in comparison to wild-type animals, indicating that dopamine synthesis was unchanged. Similar results were obtained by measuring the tissue content of dopamine and its metabolites from the striatum (Figure 6).

To determine whether the failure to observe altered dopamine transmission in animals with increased motor activity is unique to *Nr1*^{neo} −/− animals, microdialysis studies were carried out on wild-type and *Nr1*^{neo} −/− animals following treatment with MK-801. This treatment did not alter levels of striatal extracellular dopamine or dopamine metabolites in wild-type or *Nr1*^{neo} −/− mice (data not shown). Taken together, these results suggest that hyperlocomotion and stereotypy can be produced in the absence of increased dopaminergic tone.

Nr1^{neo} −/− Mice Have Social Deficits that Can Be Treated with Clozapine

Social withdrawal was apparent in adult *Nr1*^{neo} −/− mice housed with wild-type littermates. *Nr1*^{neo} −/− mice often did not sleep piled in a nest with cagemates, remaining physically distant (Figure 7). The extent of this social withdrawal was quantified using a resident–intruder behavioral assay, which can be used to measure "approach-oriented behavior" and social withdrawal (Corbett et al., 1993; Dixon et al., 1994) (Figure 7). In this assay, a resident male mouse is housed alone for at least a week and then a group-housed intruder male is added to the resident cage. Typically, the resident male actively initiates social investigation of the intruder, rarely avoiding social interaction, and sometimes initiating fights.

The amount of time that wild-type or *Nr1*^{neo} −/− residents spent engaged in social investigation, social avoidance, or fighting behaviors was monitored over a 6 min period (Figure 7). *Nr1*^{neo} −/− residents displayed significant reductions in social investigation relative to wild-type mice. Furthermore, *Nr1*^{neo} −/− residents exhibited escape behaviors, actively avoiding interaction with intruder males. In contrast, wild-type residents rarely displayed escape behavior. Administration of clozapine (0.5 mg/kg, i.p.) to *Nr1*^{neo} −/− mice 1 hr prior to testing resulted in a general increase in social investigation and a significant decrease in escape behavior (Figure 7).

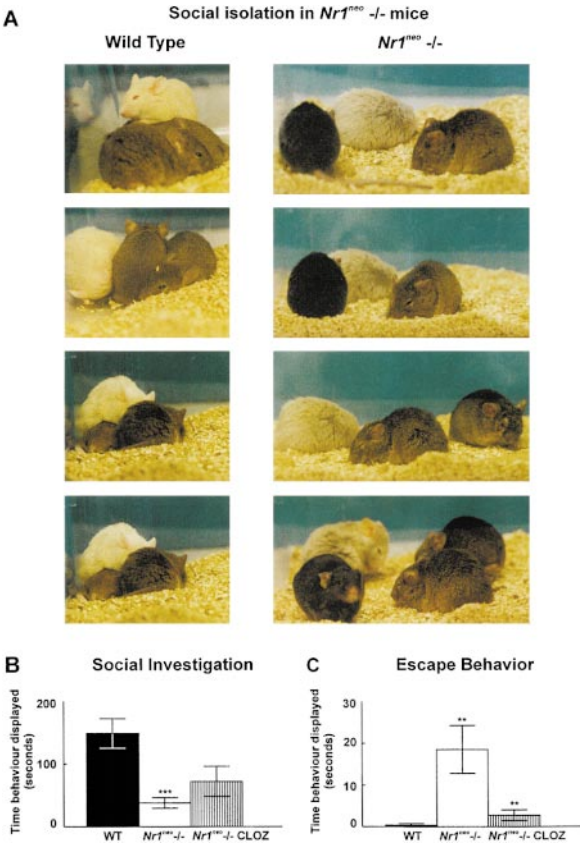

Figure 7. *Nr1*^{neo} −/− Mice Display Social Withdrawal that Can Be Quantified with the Resident–Intruder Behavioral Assay

(A) *Nr1*^{neo} −/− mice avoid contact with cagemates. Photographs of three wild-type mice housed together (left panel) and four *Nr1*^{neo} −/− mice housed together (right panel) were taken every half-hour for 2 hr. While wild-type mice typically prefer to nest, *Nr1*^{neo} −/− mice sleep isolated from other mice in the cage.

(B) Time spent by the resident male over a 6 min period actively pursuing social investigation of the intruder mouse, before and after treatment of *Nr1*^{neo} −/− mice with clozapine (0.5 mg/kg. i.p.).

(C) Time spent by the resident male over a 6 min period actively avoiding social interaction (escape behavior), before and after treatment of *Nr1*^{neo} −/− mice with clozapine (0.5 mg/kg. i.p.).

For (B) and (C), *** = $p < 0.0005$, ** = $p < 0.005$, Student's two-tailed t test. Error bars demonstrate the standard error of the mean (± SEM), n = 8 for all groups.

Sexual Dysfunction of *Nr1*^{neo} −/− Mice

Nr1^{neo} −/− males housed with wild-type females failed to produce litters even after extended breeding. Histological examination of the *Nr1*^{neo} −/− testes revealed that all stages of spermatogenesis were represented and that mature spermatozoa were present in the lumen of seminiferous tubules (data not shown). We studied the frequency with which *Nr1*^{neo} −/− mice copulate with females by presenting *Nr1*^{neo} −/− and wild-type males with females induced to ovulate by treatment with hCG (Table 1). Successful mating was scored the following morning by examination of the females for the presence of a copulation plug. While the majority of wild-type males produced copulation plugs, *Nr1*^{neo} −/− males produced none. To determine whether this abnormal sexual behavior was related to the observed social withdrawal

Table 1. Clozapine Improves the Fertility of *Nr1*^{neo}−/− Males

	Mating Frequency without Clozapine	Mating Frequency with Clozapine	χ^2 Analysis p Value
Nr1^{neo}+/+	84% (16/19)	100% (7/7)	0.5354
Nr1^{neo}−/−	0% (0/18)	30% (7/23)	0.0153

Mating frequency determined as the number of times that copulation plugs were identified from superovulated females mated with the individually housed male. Clozapine was administered at a dose of 0.5 mg/kg interperitoneally to males just prior to the addition of the female. χ^2 analysis compares untreated and clozapine-treated males for each genotype.

of *Nr1*^{neo} −/− mice, clozapine (0.5 mg/kg) was administered to males prior to mating. Following clozapine administration, *Nr1*^{neo} −/− males mated with ovulating females approximately 30% of the time, as evidenced by the production of copulation plugs (Table 1). Successful copulation was verified by examination of the females for the presence of blastocyst stage embryos 3 days after mating.

Discussion

We describe here the development of a mouse line that expresses 5%–10% of normal levels of the NR1 subunit of NMDA receptors. This level of expression is sufficient to allow survival to adulthood. NMDA receptor–deficient mice display an increase in both motor activity and stereotypic behavior similar to that observed on treatment of normal mice with the NMDA receptor antagonists PCP or MK-801 but are themselves resistant to the stimulatory effects of these drugs. Treatment with either of the antipsychotic drugs haloperidol or clozapine ameliorated the increased locomotion and stereotypy observed in these mice. In addition to altered motor activity, NMDA receptor–deficient mice display deficits in sexual and social behavior. These behavioral deficits can also be improved by treatment with clozapine.

The NR1-deficient mice studied here were maintained on a mixed genetic background consisting of alleles derived from 129/Ola, C57BL/6, and DBA/2. The importance of the genetic background on which mutations in specific genes are studied has been extensively described in recent reviews and commentaries (Branbury Conference, 1997; Crawley et al., 1997). To increase the probability that alleles of various genes, including those linked to *Nr1*, were included in both populations, mice that had inherited the wild-type 129 allele from the ES chimeras were used in establishing the breeding population. However, while all *Nr1*^{neo} −/− mice were expected to be homozygous for 129 alleles of genes linked to *Nr1*, this would be true of only a quarter of the control animals. We cannot, therefore, formally rule out the possibility that homozygosity at these loci contributes in part to the phenotype described. This seems unlikely, however, since previous studies have reported that 129 mice display slightly less motor activity than C57BL/6 and DBA/2 (Kelly et al., 1998). Furthermore, strain-specific alleles were inherited equally in *Nr1* +/+ and −/− mice, based on the examination of 11 microsatellite marker genes on ten chromosomes (data not shown).

The hypomorphic mutation introduced into the *Nr1*

gene allows sufficient expression to avoid perinatal lethality, thus allowing the consequences of chronic reduction in expression of all NMDA receptors to be studied for the first time in adult animals. The genetic reduction of NMDA receptors results in a number of behaviors that have been traditionally characterized as modeling behaviors associated with schizophrenia, and these behaviors are reversed with antipsychotic drugs. Increased motor activity and stereotypy are the defining features of current pharmacological models of schizophrenia (Carlsson and Carlsson, 1990; Corbett et al., 1995), and the amelioration of these behaviors is the standard screening test for the efficacy of antipsychotic drugs (Carlsson and Carlsson, 1990; Corbett et al., 1995; Moghaddam and Adams, 1998). Hyperlocomotion has been correlated with the positive symptoms of schizophrenia, whereas social withdrawal, such as is observed in the *Nr1*^{neo} −/− mice, has been correlated with negative symptoms of the disease (Corbett et al., 1993; Dixon et al., 1994).

The infertility of *Nr1*^{neo} −/− males may provide a model for another deficit observed in schizophrenia, as schizophrenic males have a significant reduction in fertility (Odegard, 1980; Saugstad, 1989). This reduction has been attributed to a reduced ability to establish sexual relationships (Nimgaonkar et al., 1997) rather than to any physiological abnormality. Our studies of the *Nr1*^{neo} −/− mice suggest that the observed infertility also results from changes in behavior, since it can be improved by treatment with clozapine. Clozapine has been reported to increase "approach-oriented behavior" and reduce social withdrawal in mice (Dixon et al., 1994). This drug may therefore restore fertility by allowing males to overcome their social indifference. In contrast to the infertility observed in NMDA receptor–deficient mice, alterations in sexual behavior have not been reported for pharmacological models of schizophrenia. This difference may reflect the difficulty in studying complex behaviors, such as mating, in models where behavior resembling schizophrenia is relatively short lived and underscores the potential usefulness of a model in which chronic reduction in NMDA receptor activity is achieved using a genetic approach.

Schizophrenia is regarded as a progressive mental illness because symptoms such as cognitive impairment worsen with age and early diagnosis and treatment have been associated with an improved outcome (Coyle, 1996). Future studies should allow us to determine whether the deficits in motor activity and social behavior seen in the *Nr1*^{neo} −/− also become more severe with age. In addition, as long-term administration of clozapine is required for optimal treatment of schizophrenia (Meltzer, 1991), it is possible that extended administration of clozapine will further improve the behavioral deficits seen in the *Nr1*^{neo} −/− mice.

Hyperlocomotion and stereotypy are commonly considered behaviors of increased dopaminergic tone (Giros et al., 1996; Gainetdinov et al., 1999), and most of the hypotheses concerning the motor stimulatory effects of PCP or MK-801 suggest that these drugs either directly or indirectly increase dopaminergic tone (Imperato et al., 1990; Miller and Abercrombie, 1996). Such studies suggest that NMDA receptor blockade results in the disinhibition of nigrostriatal dopaminergic neurons, resulting in an indirect increase in dopaminergic tone. However, this hypothesis is not consistent with our failure to detect significant changes in striatal dopaminergic parameters. The results obtained from the *Nr1* mutant mice are more consistent with an alternate model proposed by Carlsson and colleagues (1997) in which glutamatergic and dopaminergic neurons share common postsynaptic targets in the striatum with opposing effects. In this model, glutamate exerts an excitatory influence on these target neurons, while dopamine exerts an inhibitory influence (Carlsson et al., 1997). These target neurons in turn are proposed to have an inhibitory action on the expression of motor behaviors. This model provides an explanation for our observation that hyperlocomotion in *Nr1*^{neo} −/− mice does not result from increased dopaminergic tone.

We have demonstrated that while both typical and atypical antipsychotics can attenuate hyperlocomotion and stereotypy in the *Nr1*^{neo} −/− mice, clozapine is more effective than haloperidol. Clozapine significantly improved abnormal behavior at doses that had minimal effects in wild-type mice, while haloperidol was effective only at doses that also decreased the activity of wild-type animals. These results are in agreement with previous pharmacological studies (Martin et al., 1998) and are consistent with the interpretation that the increased motor activity of *Nr1*^{neo} −/− mice is not due to increased dopaminergic tone. Clozapine, which also has a significant 5HT2$_A$ antagonist activity and interacts with H1 histamine and α-adrenergic receptors (Meltzer, 1991; Martin et al., 1998), may be acting more directly on pathways that are altered as a result of NR1 deficiency. Further characterization of *Nr1*^{neo} −/− mice may establish which of clozapine's many targets are responsible for attenuating the abnormal motor behaviors observed in these mice.

In summary, the mice described here support a model in which decreased NMDA receptor expression leads to extensive behavioral changes that are believed to model schizophrenia. Furthermore, the evidence presented here suggests that monoaminergic transmission also plays a role in these changes. The response of *Nr1*^{neo} −/− mice to antipsychotics addresses the question of how symptoms of schizophrenia can be treated with drugs that act on dopaminergic and serotonergic systems, yet be associated with a dysfunction of the glutamatergic system. Continued studies of these mice will undoubtedly lead to a better understanding of the role of NMDA receptors in this disease. In addition, these animals will provide a tool for examining the involvement of NMDA receptors in the wide range of both normal and pathological processes in which they have been proposed to play a role.

Experimental Procedures

Generation of *Nr1*^{neo} −/− Animals

Genomic clones spanning the *Nr1* locus were isolated from a 129/SvEv λ bacteriophage library (Stratagene, La Jolla) using *Nr1* cDNA exons 11–20 as a probe and used to generate the targeting construct, Nr1^{neo}. An 8.0 kb DNA fragment extending from the EcoRI site of intron 10 to the SmaI site of intron 20 was inserted 5′ of the *neo* gene at the NotI site of the vector JNS2 (Dombrowicz et al., 1993). A 2.5 kb SmaI-BamHI fragment corresponding to intron 20–

intron 21 was cloned into the XbaI and BamHI sites of JNS2 with a NheI linker (New England Biolabs, Beverly, MA). Nr1^{neo} was linearized with PvuI, electroporated into E14Tg2a ES cells (Hooper et al., 1987), and transformed cells selected in the presence of G418 and ganciclovir using methods previously described (Mohn and Koller, 1995). Targeted clones were identified by Southern analysis and injected into blastocysts to generate chimeras, which were bred to B6D2 animals to obtain *Nr1^{neo}* +/− animals. These wild-type and heterozygous animals were intercrossed to obtain *Nr1^{neo}* +/− offspring mice, which were in turn mated to establish a breeding colony. All wild-type mice and mice homozygous for the mutant *Nr1* gene were obtained from the intercross of these heterozygous animals.

Northern Blot

Total RNA was prepared using RNAzol B (TelTest, Friendswood, TX) from brains of 8-week-old mice. Twenty micrograms of total RNA of each genotype was electrophoresed on a 1.2% agarose, 1.1% formaldehyde gel and transferred to nitrocellulose membrane. The membrane was hybridized with the cDNA probe descibed above and revealed by autoradiography. The membrane was stripped and probed for mouse β-actin (Stratagene). NIH Image 1.62 Beta was used to digitally capture and analyze autoradiograpic images from the Northern blot.

Western Blot

Protein extracts were prepared from brains of 8-week-old mice of each genotype. Each brain was homogenized in a 5 ml buffer of 50 mM Tris-acetate (pH 7.4), 10% sucrose, 5 mM EDTA with 100 μl protease inhibitor (Sigma, St. Louis). Homogenates were fractionated on a sucrose gradient to enrich for plasma membranes. Crude membrane fractions of 25 μg protein were electrophoresed in a 12%–20% gradient SDS-acrylamide gel, transferred to PVDF membrane, and incubated with a 1:1000 dilution of α-NMDAR1 monoclonal antibody (clone 54.1, Pharmingen, San Diego, CA) for 1 hr at room temperature. Antibody binding was detected with ECL chemiluminescence kit (Amersham, Arlington Heights, IL). PVDF membranes were probed with an α-β tubulin monoclonal antibody (Sigma) to verify equivalent sample loading. Data representation and densitometric analysis were done using NIH Image 1.62 Beta.

[^{3}H]MK-801 Binding

Pooled tissues from the prefrontal cortex of three wild-type and *Nr1^{neo}* −/− mice were homogenized in a binding buffer of 20 mM HEPES, 1 mM EDTA (pH 7.0) with 100 μM glutamate, glycine, and spermidine as described in Nankai et al. (1996). Binding assays included 80 μg membrane protein and 2 nM [^{3}H]MK-801 in a volume of 150 μl. Tubes were incubated at 32°C for 3 hr to reach equilibrium binding. Nonspecific binding was determined by 10 μM MK-801. Bound ligand was separated from free by rapid filtration onto Whatman GF/B filters. Data were fit by nonlinear regression analysis using GraphPad Prism software.

RT–PCR of *Nr1^{neo}* −/− mRNA

Total RNA from brains of *Nr1^{neo}* −/− and wild-type mice was isolated as described above and 3 μg used as a template for reverse transcription (cDNA Cycle Kit, Invitrogen, San Diego, CA), followed by PCR amplification with primers specific for *Nr1* exons 10–22. Overlapping regions of *Nr1* cDNA were applified using three primer sets: 5′ACAGAGAAGCCTCGAGGATA with 5′AGGAAAACCACATGGCA GAG, 5′TTCAGTCCCTTTGGCCGATT with 5′GCGGGAGTCACATTC TTGAT, and 5′GGGTACTCTTACCGAAGTAC with 5′AGAAATACACA GACAAGGCG. The PCR products were cloned into pCR2.1 using the TA cloning kit (Invitrogen). For each PCR product, two independent clones were sequenced by Taq cycle sequencing using an Applied BioSystems 373 A Automated Sequencer (UNC-CH Automated Sequencing Facility).

Pharmacological Agents

Drugs were prepared fresh in saline and administered intraperitoneally in a volume of 0.1 ml/10 g body weight. MK-801 and PCP (Research Biochemicals Inc., Natick, MA) were dissolved in saline, while clozapine (Reseach Biochemicals Inc.) and haloperidol (Sigma) were first dissolved in a drop of glacial acetic acid. Pharmacological agents were injected just prior to monitoring motor activity.

Analysis of Motor Activity

Nr1^{neo} −/− animals and their wild-type littermates were obtained as the F2 generation of *Nr1^{neo}* +/− intercrosses. Animals were individually tested for motor activity at 10–14 weeks of age under standardized environmental conditions using an automated Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, OH), in a 42 cm^2 plexiglass chamber. Horizontal activity, measured as the total distance traveled by each mouse, and stereotypy, measured as repetitive movements with an interval less than 1 s, were recorded in 5 min intervals over a 2 or 4 hr period, and the raw numbers were averaged to give values for each 5 min interval (Gainetdinov et al., 1999). For all data presented in this paper, values represent means ± SEM, and statistical significance was analyzed by two-way ANOVA and by two-tailed Student's t test on 2 hr summed data. All experiments were conducted in accordance with the NIH guidelines for the care and use of animals and with approved animal protocols from the University of North Carolina at Chapel Hill Animal Care and Use Committee and the Duke University Animal Care and Use Committee.

Assessment of Brain Dopamine Content

Tissue Content

The striata from four adult wild-type or *Nr1^{neo}* −/− mice were dissected and homogenized in 0.1 M $HClO_4$ containing 100 ng/ml 3,4-dihydroxybenzylamine (DHBA). Following centrifugation at 10,000 × g for 10 min, the supernatants were filtered through 0.22 μm filters and analyzed for the levels of dopamine, DOPAC, and HVA by HPLC-EC (Wang et al., 1997).

Quantitative "Low Perfusion Rate" Microdialysis

Microdialysate samples were collected from the right striatum 24 hr after surgery, separated, and quantified by HPLC as described for freely moving mice (Wang et al., 1997). Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.), placed in a stereotaxic instrument, and implanted with dialysis probes (2 mm membrane length, 0.24 mm o.d.; Cuprophane, 6 kDa cut-off; CMA/Microdialysis, Solna, Sweden) using CMA-11 guide cannulae into the right striatum (AP: 0.0, DV: −4.4, and L: 2.5 mm relative to bregma for both wild-type and *Nr1^{neo}* −/− mice) (Franklin and Paxinos, 1996). Following surgery animals were returned to their cages and given free access to food and water. Twenty-four hours after surgery, the dialysis probe was connected to a syringe pump and perfused at 70 nl/min with artificial CSF (Na^+ 150 mM; K^+ 3.0 mM; Ca^{2+} 1.4 mM; PO_4^- 31.0 mM; Cl^- 155 mM [pH 7.3]; with 0.25 mM ascorbate). After a 2 hr equilibration period, perfusates were collected every 90 min in a tube containing 2 μl of 0.4 M $HClO_4$. Levels of dopamine and metabolites were quantified using an HPLC-EC as described in Wang et al., 1997.

Resident–Intruder Assay

Male wild-type or *Nr1^{neo}* −/− mice were housed individually or in groups of three or four for at least 1 week prior to testing, and cage bedding was changed 24 hr prior to testing. As described in Dixon et al. (1994), in this assay, a group-housed male (intruder) was placed in the home cage of the individually housed male (resident), and their behavior was video recorded for 6 min. Experimental groups included eight wild-type residents, eight *Nr1^{neo}* −/− residents, eight wild-type intruders, and eight *Nr1^{neo}* −/− intruders. One week after the initial set of experiments, the mice were retested under identical conditions, except that *Nr1^{neo}* −/− mice were injected with 0.5 mg/kg (i.p.) clozapine 1 hr prior to testing. The videotaped behavior of the resident or intruder male was individually scored by two blinded observers for time spent in social investigation (approaching, sniffing, grooming other mouse, and sexual behaviors), in escape behaviors (actively avoiding other mouse), or in fighting behaviors. The scores for each behavior were subsequently averaged for each genotype.

Assessment of Mating Behavior

Wild-type or *Nr1^{neo}* −/− male mice aged 10–15 weeks were individually housed and mated with a 3.5-week-old C57BL/6J female that had been treated with pregnant mare serum and human chorionic

gonadotropin to induce ovulation (Masters and Wheeler, 1996). Matings were initiated at 4:00 to 5:00 p.m., and the female mice were examined for the presence of copulation plugs on the following morning. A set of four wild-type males and six mutant males were mated with ovulating females two to three times a week for 2 weeks. These males were subsequently used for a second group of matings identical to the first in design except that 0.5 mg/kg clozapine was administered (i.p.) just prior to mating with females. Plug-positive females (Rosenbauer et al., 1980) were checked 3 days later for the presence of blastocyst embryos in the uterine horns to verify that the males had mated with the females and that they were fertile.

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