

Genetic Inactivation of the NMDA Receptor NR2A Subunit has Anxiolytic- and Antidepressant-Like Effects in Mice

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There is growing evidence implicating the glutamate system in the pathophysiology and treatment of mood and anxiety disorders. Glutamatergic neurotransmission is mediated by several receptor subfamilies including multiple NMDA receptor subunits (NR2A-D). However, little is currently understood about the specific roles of NMDA subunits in the mediation of emotional behavior due to a lack of subunit-specific ligands. In the present study, we employed a mouse gene-targeting approach to examine the role of the NR2A subunit in the mediation of anxiety- and depressive-related behaviors. Results showed that NR2A knockout (KO) mice exhibit decreased anxiety-like behavior relative to wild-type littermates (WT) across multiple tests (elevated plus maze, light–dark exploration test, novel open field). NR2A KO mice showed antidepressant-like profiles in the forced swim test and tail suspension test, as compared to WT controls. Locomotor activity in the nonaversive environments of the home cage or a familiar open field were normal in the NR2A KO mice, as were gross neurological and sensory functions, including prepulse inhibition of startle. Taken together, these data demonstrate a selective and robust reduction in anxiety- and depression-related behavior in NMDA receptor NR2A subunit KO mice. Present results support a role for the NR2A subunit in the modulation of emotional behaviors in rodents and provide insight into the role of glutamate in the pathophysiology and treatment of mood and anxiety disorders.

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INTRODUCTION

There is growing evidence in support of an important role for the glutamate system in the mediation of emotion, and in the pathophysiology of mood and anxiety disorders. Some but not all studies have found altered levels of glutamate and glutamate receptor binding in the brains of depressed patients and suicide victims (Auer *et al*, 2000; Holemans *et al*, 1993; Law and Deakin, 2001; Meador-Woodruff *et al*, 2001; Nowak *et al*, 1995; Palmer *et al*, 1994; Pflieger *et al*, 2003). In rodents, exposure to stress produces a robust increase in glutamate release in regions mediating emotional behavior, such as the locus coeruleus, hippocampus and prefrontal cortex (Moghaddam, 1993; Moghaddam *et al*, 1994; Singewald *et al*, 1995, 1996). Additionally, exposure to stress increases NMDA receptor

expression and mRNA levels in the rat ventral tegmental area and hippocampus (Bartanusz *et al*, 1995; Fitzgerald *et al*, 1996).

While these data point to glutamatergic involvement in emotional regulation, defining this role is difficult because glutamate exerts its effects on neural function in a highly complex manner. Glutamatergic neurotransmission is mediated via ionotropic (*N*-methyl-D-aspartate (NMDA), L-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionat (AMPA), kainate) and metabotropic receptors. However, possible differential functional roles of these receptor subfamilies in the mediation of behavioral processes such as emotion remains incompletely understood. Recent studies in rodents have demonstrated that blockade of glutamate's actions at metabotropic and AMPA receptors exerts antidepressant-like and anxiolytic-like effects in various tasks, leading to the clinical development of metabotropic glutamate receptors for emotional disorders (for reviews, see (Javitt, 2004; Spooren and Gasparini, 2004; Swanson *et al*, 2005)). Manipulation of NMDA receptor function has also been found to affect emotion-related behaviors in rodent models. In rats, treatment with competitive NMDA receptor antagonists produces anxiolytic- and antidepressant-like effects in various tasks, such as the elevated plus-maze and forced swim test, at doses that

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do not produce nonspecific changes in locomotor activity (Martinez *et al*, 2002; Molchanov and Guimaraes, 2002; Przegalinski *et al*, 2000; Trullas and Skolnick, 1990; Wiley *et al*, 1995). Moreover, some but not all, noncompetitive NMDA receptor channel blockers exert anxiolytic- and antidepressant-like effects in these models (Silvestre *et al*, 1997; Wiley *et al*, 1995).

While these findings suggest NMDA receptor modulation of emotional behaviors, it remains unclear whether these effects result from antagonism of specific NMDA receptor subunits. NMDA receptors are tetramers composed of two NR1 and two NR2 (NR2A-NR2D) subunits (Laube *et al*, 1998; Rosenmund *et al*, 1998; Schorge and Colquhoun, 2003). The NR2 subunit present in a functional NMDA receptor determines the physiological characteristics of the NMDA receptor; for example, NMDA receptors containing NR2A mediate faster neurotransmission than NR2B-containing NMDA receptors (Cull-Candy *et al*, 2001). In addition, NR2A and NR2B subunits are differentially expressed over the course of development, with NR2B predominating in the mouse brain until the end of the second postnatal week (Liu *et al*, 2004b). In the adult mouse brain, NR2A- and NR2B-containing NMDA receptors are both highly expressed in brain regions implicated in emotion (Law *et al*, 2003; Magnusson *et al*, 2002; Sah and Lopez De Armentia, 2003). Determining the differential roles for specific subunit-containing receptors in behavior is hampered by the lack of NR2A subunit selective pharmacological ligands. In this context, gene-targeting in mice provides a valuable approach to studying the molecular basis of emotional behavior (Holmes, 2001; Holmes *et al*, 2003a,b,c), and the behavioral functions of NMDA subunits (Boyce-Rustay and Holmes, 2005; Tang *et al*, 1999; Tsien *et al*, 1996).

In the current study, we used a mouse model in which the gene encoding the NR2A subunit (*Grin2a*) was functionally inactivated to investigate the role of NR2A in regulating emotional behavior. Homozygous NR2A null mutant ('knockout,' KO) and heterozygous mutant (HET) mice were compared to wild-type (WT) littermate controls on a battery of tests for anxiety-related behaviors (elevated plus maze, light/dark exploration test, novel open field) and depression-related behaviors (forced swim test, tail suspension test). To control for nonspecific phenotypic alterations that could confound interpretation of anxiety- and depression-related behaviors in NR2A KO mice, such as motoric abnormalities (Holmes and Cryan, 2005), NR2A KO mice were assessed for locomotor activity in nonaversive environments (home cage, familiar open field) and examined on a screen for gross behavior, neurological and sensory functions (Crawley and Paylor, 1997; Holmes *et al*, 2001).

MATERIALS AND METHODS

Subjects

NR2A KO mice were generated as previously described (Sakimura *et al*, 1995; Watanabe *et al*, 1993). Briefly, the targeting vector (pTVGR ϵ 1) was a 1.3 kb blunted *EcoRI*-*BamHI* fragment containing the neomycin phosphotransferase gene under the control of the phosphoglycerate

kinase promoter. This was then inserted into the *XcmI* site of the 10.0 kb *GluR ϵ 1* DNA from a C57BL/6 genomic library and a synthetic adaptor carrying *SaI*, *HindIII* and *SmaI* sites replaced *NotI*-*XhoI* segment (16.5 kb) located between the *ClaI* and *SacI* sites. DNA was digested with *NotI*. TT2. C57BL/6 \times CBA F1 hybrid ES cells were cultured, selected, and the target clones were identified by G418 selection, PCR and South blot hybridization. Embryonic stem cell clones were microinjected into ICR blastocysts to obtain chimeric progeny. Chimeric males were mated to C57BL/6 female mice. Offspring were genotyped by Southern blot analysis of tail biopsies to confirm germline transmission.

For the current study the NR2A null mutation was backcrossed into the C57BL/6J strain for >10 generations to produce a congenic C57BL/6J genetic background. Analysis of 150 SNP markers at ~15–20 megabase intervals across all autosomal chromosomes confirmed >99% C57BL/6J congenicity in the mutant line (JRS Allele Typing Services, The Jackson Laboratory, Bar Harbor, ME, USA). To avoid potential phenotypic abnormalities resulting from genotypic differences in maternal behavior and early life environment, NR2A KO, NR2A HET and WT mice were all generated from HET \times HET matings. Mice were bred and maintained at The Jackson Laboratory (Bar Harbor, ME, USA) and shipped to the NIH at 7–9 weeks of age and testing began at a minimum of 10 weeks of age. On arrival at NIH, mice were housed in groups of 2–4 with same-sex littermates in a temperature and humidity controlled vivarium under a 12 h light/dark cycle (lights on 06:00).

Behavioral Testing

Testing was conducted using five separate cohorts of mice. One cohort (11 male WT, three female WT, 12 male HET, six female HET, seven male KO, seven female KO) was evaluated for physical health, sensory and neurological functions, and then tested on the novel open field, elevated plus maze, light–dark exploration, and tail suspension test, in that order, with at least 1 week between tests. A second cohort (second male WT, four female WT, two male HET, four female HET, two male KO, one female KO) was tested on the forced swim test. A third cohort (six male WT, four female WT, six male HET, six female HET, six male KO, six female KO) was tested on the forced swim test and home cage activity with >10 weeks between experiments. A fourth cohort (six male WT, six female WT, seven male HET, eight female HET, 11 male KO, four female KO) was tested for acoustic startle and prepulse inhibition of the startle response. A fifth cohort (four male WT, four female WT, six male HET, five female HET, six male KO, three female KO) was tested on the repeated open field test. With the exception of the home activity, all testing was conducted during the light phase of the light/dark cycle. Where appropriate, apparatuses were cleaned between subjects with a 70% ethanol (v/v) solution. The experimenter remained blind to genotype during testing; animals were identified by subcutaneously implanted microchips. All experimental procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee and strictly followed the NIH guidelines 'Using Animals in Intramural Research'.

Tests for Anxiety-Related Behaviors

Elevated plus-maze. The elevated plus-maze test was conducted as previously described (Handley and Mithani, 1984; Holmes *et al.*, 2003a, c). The apparatus consisted of two open arms (30 × 5 cm; 90 lux) and two closed arms (30 × 5 × 15 cm; 20 lux) extending from a 5 × 5 cm central area and elevated 20 cm from the ground (San Diego Instruments, San Diego, CA, USA). The walls were made from black ABS plastic and the floor from white ABS plastic. A 0.5 cm raised lip around the perimeter of the open arms prevented mice from falling off the maze. The mouse was placed in the center facing an open arm and allowed to explore the apparatus for 5 min. Time spent in the open arms, and entries into the open and closed arms were measured by the Ethovision videotracking system (Noldus Information Technology Inc., Leesburg, VA, USA).

Light-dark exploration test. The light-dark exploration test was conducted as previously described (Crawley, 1981; Holmes *et al.*, 2003a, c). The apparatus consisted of two compartments (each 17 × 13 × 13 cm), one with white Plexiglas walls and clear Plexiglas floor (40 lux) ('light' compartment) and the other with black Plexiglas walls and clear Plexiglas floor (0 lux) ('dark' compartment) that were connected by a partition at floor level with a small opening (5 cm) (Med Associates, Georgia, VT, USA, Model ENV-3013). The mouse was placed into the dark compartment facing away from the aperture and allowed to explore the apparatus for 10 min. Time spent and full-body transitions into the light compartment, and total full-body transitions between the light and dark compartments were measured by photocells connected to Med Associates software.

Novel and familiar open field tests. The novel open field test was conducted as previously described (Holmes *et al.*, 2003a, c). The apparatus was a 40 × 40 × 35 cm square arena (50 lux) constructed of white Plexiglas. The mouse was placed in the perimeter and allowed to explore the apparatus for 60 min. Total distance traveled in the whole arena and time spent in the center (20 × 20 cm) was measured by the Ethovision videotracking system (Noldus Information Technology Inc., Leesburg, VA, USA).

In a separate experiment to compare responses to a novel vs familiar open field, open field-naïve mice were exposed to the open field for 30 min per day on 5 consecutive days, using the same apparatus and procedure as described above.

Home cage locomotor activity. Home cage activity was measured to assess locomotor behavior in a non-aversive environment, as described previously (Holmes *et al.*, 2002a). Mice were individually housed in a standard home cage under normal vivarium conditions and left undisturbed for a 48 h acclimation period. Home cage activity was then measured over 24 h using the photocell-based Opto M3 activity monitor (Columbus Instruments, Columbus, OH, USA).

Tests for Depression-Related Behaviors

Forced swim test. The forced swim test was conducted as previously described (Holmes *et al.*, 2002b; Porsolt *et al.*,

1978). The apparatus was a transparent Plexiglas cylinder (20 cm diameter) filled halfway with water (24 ± 1 °C). The mouse was gently lowered into the water and manually observed for the presence/absence of immobility (cessation of limb movements except minor involuntary movements of the hind limbs) every 5 s during the last 8 min of a 10 min test session.

Tail suspension test. The tail suspension test was conducted as previously described (Holmes *et al.*, 2002b; Steru *et al.*, 1985). The mouse was securely fastened by the end of the tail to a flat surface that was suspended in a visually isolated white Plexiglas box (40 cm³) and manually observed for the presence/absence of immobility (cessation of limb movements) every 5 s during the last 4 min of a 6 min session.

Evaluation of Empty Cage Behaviors, Physical Health, Sensory, and Neurological Functions

Mice were evaluated for empty cage behaviors, physical health, sensory and neurological functions using methods based on those described previously (Crawley and Paylor, 1997; Holmes *et al.*, 2001). Mice were first placed in a bare empty home cage and observed for the presence of freezing, trembling, sniffing, licking, rearing, jumping, spontaneous seizure, defecation, urination, head bobbing, circling, abnormal gait, and retropulsion over 60 s. Basic physical health was then evaluated by examining for missing whiskers, bald patches, exophthalmus, straub tail, kinked tail, kyphosis, lordosis, body weight and core body temperature. Simple sensory reflexes were measured via orienting responses to an approaching hand and to physical touch, and *via* palpebral closure on touch of the eye, twitch of the pinna on touch and an orienting response to tail pinch. Basic motor and neurological function was assessed by observing for splayed limbs, forepaw clutch and hind limb clutch when mice were suspended upside down by the tail.

Mice were assessed for acoustic startle and prepulse inhibition of the startle response as previously described (Holmes *et al.*, 2001) using 4 SR-Lab System startle chambers (San Diego Instruments, San Diego, CA, USA). Mice were placed into clear Plexiglas cylinders for a 5 min acclimation period. A 65 dB broadband background noise was presented during acclimation and throughout the test session. During the test session, mice were presented with startle trials (40-msec 120-dB broadband sound pulse) and prepulse + startle trials (20-ms noise prepulse sound followed, 100 ms later, by a 40-msec 120-dB broadband sound pulse). The prepulse + startle trials were preceded and followed by five pulse alone trials, which were not included in the analyses. Test trials consisted of 10 trials of three different intensities (3, 6 and 12 dB above background). Each trial type was presented 10 times with a variable interval of 12–30 s between each presentation. Basal activity in the startle chambers was measured during no-stimulus trials. Startle amplitude was measured every 1 ms, over a 65 ms period beginning at the onset of the startle stimulus. The peak startle amplitude over the sampling period was taken as the dependent variable. Whole-body startle responses were measured *via* vibrations transduced into

analog signals by a piezoelectric unit attached to the platform on which the cylinders rested. Prepulse inhibition of startle was calculated as $100 - ((\text{startle response for prepulse} + \text{startle trials} / \text{startle response for startle-alone trials}) \times 100)$.

Statistical Analysis

Analysis of all data using sex as a factor found no genotype \times sex interactions; therefore data were collapsed across sex. Genotype effects were analyzed by use of analysis of variance (ANOVA) and Neuman-Keuls *post hoc* tests where appropriate. The effects of genotype and time (novel open field test), and genotype and trial (novel vs familiar open field test), on locomotor activity in the open field were measured by use of a 2-factor ANOVA, with repeated measures for time and trial, respectively, followed by Neuman-Keuls *post hoc* tests. Analyses were conducted using StatView (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $P < 0.05$.

RESULTS

Reduced Anxiety-Like Behavior in the Elevated Plus-Maze

There was a significant effect of genotype on measures of anxiety-related behavior, but not general locomotor activity, in the elevated plus-maze. As shown in Figure 1, NR2A KO, but not HET, mice spent significantly more time (main effect of genotype: $F_{2,43} = 7.30$, $P < 0.01$) and made significantly more entries into the open arms (main effect of genotype: $F_{2,43} = 6.01$, $P < 0.01$) as compared to WT controls. There were no genotype differences in the number of closed arm entries ($P > 0.05$).

Reduced Anxiety-Like Behavior in the Light-Dark Exploration Test

Genotype significantly affected measures of anxiety-related behavior in the light/dark exploration test. NR2A KO, but not HET, mice spent significantly more time (main effect of genotype: $F_{2,43} = 6.82$, $P < 0.01$) and made significantly more entries (main effect of genotype: $F_{2,43} = 6.39$, $P < 0.01$) into the light compartment as compared to WT controls (Figures 2a and b). NR2A KO mice also made significantly more light-dark transitions (main effect of genotype: $F_{2,43} = 6.37$, $P < 0.01$) as compared to WT controls (Figure 2c).

Reduced Anxiety-Like Behavior in the Novel Open Field Test

There was a significant effect of genotype on behavior in the novel open field. Results in Figure 3 show that NR2A KO, but not HET, mice traveled a significantly greater distance than WT controls overall (main effect of genotype: $F_{2,473} = 12.26$; $P < 0.01$). All genotypes showed differential rates of locomotor habituation over the course of the 60 min test session (main effect of time: $F_{11,473} = 70.49$, $P < 0.01$; genotype \times time interaction: $F_{22,473} = 3.58$, $P < 0.01$). *Post hoc* analysis revealed that NR2A KO mice exhibited increased levels of locomotor activity during the start of

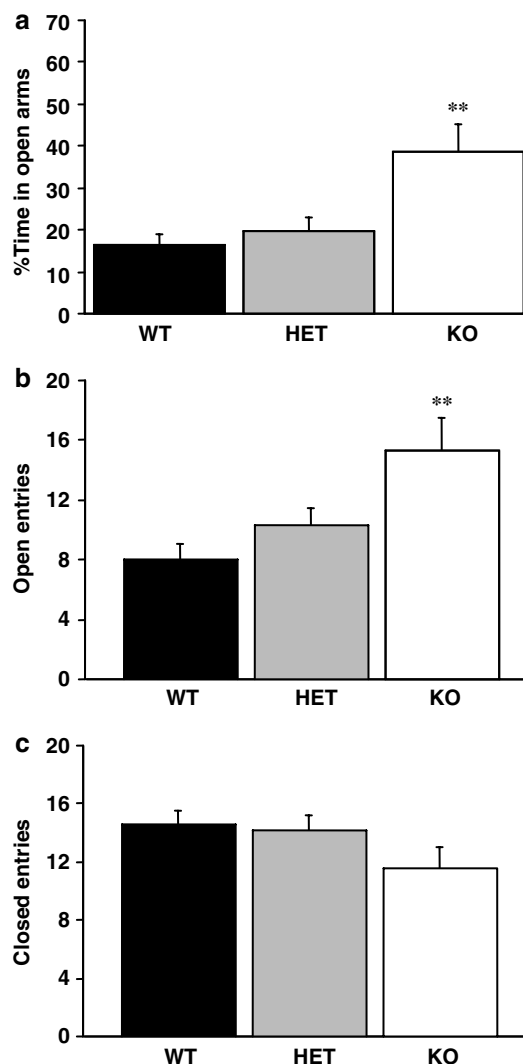


Figure 1 NR2A KO mice exhibit reduced anxiety-like behavior in the elevated plus-maze. NR2A KO mice spent more time (a) and made more entries (b) into the open arms, but made a similar number of entries into the closed arms (c), as compared to wild-type controls (WT) ($n = 14-18$ /genotype). ** $P < 0.01$ vs WT. Data in Figures 1-5 are mean \pm SEM.

the session but were no different from WT controls by the final 5 min time bin. Overall, both NR2A KO and HET mice spent a significantly greater percentage of time in the center of the open field than WT controls (main effect of genotype: $F_{2,43} = 5.19$; $P = 0.01$).

Normal Locomotor Activity in a Familiar Open Field

Altered open field locomotor activity in NR2A KO mice was dependent upon the novelty of the open field. Figure 4a shows that genotype differences in total distance traveled became successively lesser with repeated daily exposures to the open field, such that locomotor activity was no different between NR2A KO and WT by the fourth trial, while NR2A HET mice showed significantly less total distance traveled than WT and NR2A KO mice on trial 4 (main effect of genotype: $F_{2,100} = 10.16$, $P < 0.01$; main effect of day $F_{4,100} = 35.14$, $P < 0.01$; genotype \times day interaction: $F_{8,100} = 2.81$, $P < 0.01$). In contrast, NR2A KO mice spent

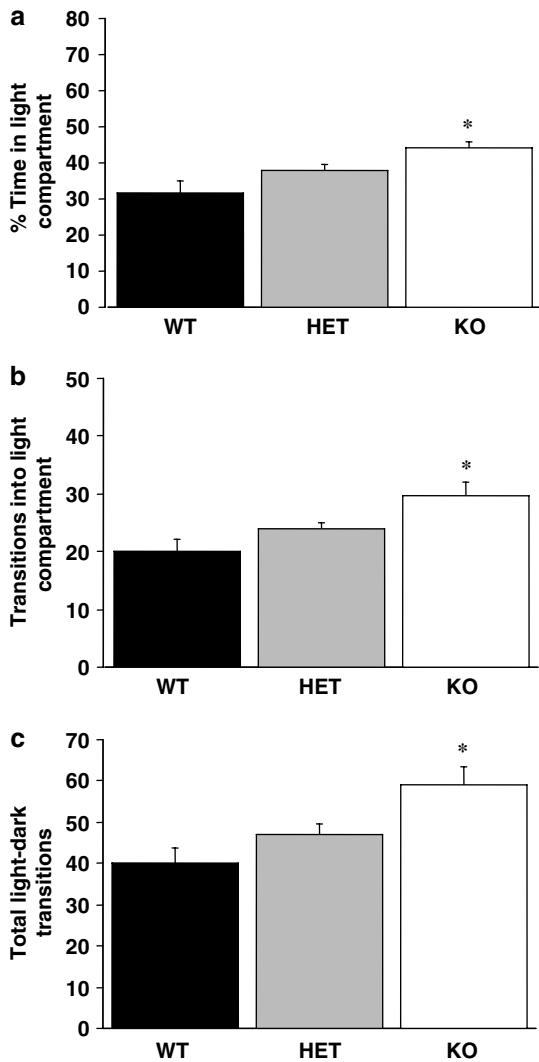


Figure 2 NR2A knockout (KO) mice exhibit reduced anxiety-like behavior in the light/dark exploration test. NR2A KO mice spent more time (a) and made more transitions (b) into the light compartment, and made more light–dark transitions overall (c), as compared to wild-type controls (WT) ($n = 14–18/\text{genotype}$). * $P < 0.05$ vs WT.

a significantly greater percentage of time in the center of the open field than WT controls on trials, either when locomotor activity was different (trials 1–3) or no different (trial 4) between genotypes (main effect of genotype: $F_{2,100} = 7.50$, $P < 0.01$; main effect of day: $F_{4,100} = 12.56$, $P < 0.01$; genotype \times day interaction: $F_{8,100} = 3.43$, $P < 0.01$) (Figure 4b). NR2A HET showed significantly more center time than WT controls on the first trial.

Normal Locomotor Activity in the Home Cage

Consistent with the normal locomotor activity phenotype in NR2A KO mice in a familiar open field, there were no genotype differences in 24 h locomotor activity in the home cage (main effect of genotype: $P > 0.05$) (Figure 4c).

Antidepressant-Like Phenotype in the Forced Swim Test

There was a significant effect of genotype on behavior in the forced swim test for depression-related behavior. As shown

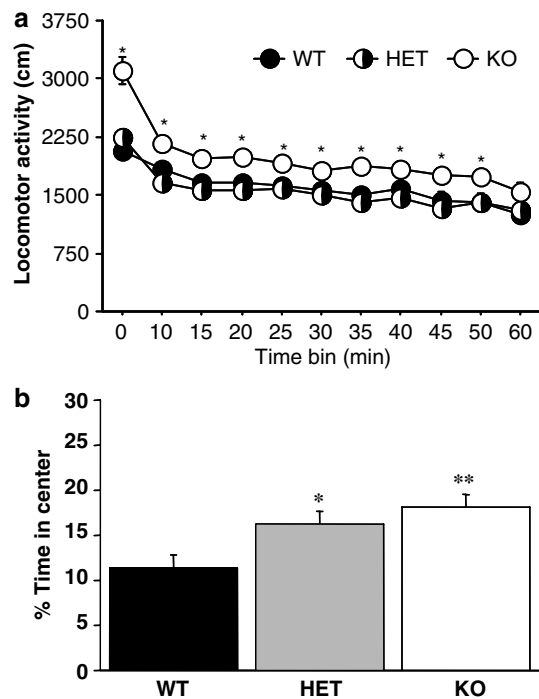


Figure 3 NR2A knockout (KO) mice exhibit reduced anxiety-like behavior in the novel open field test. (a) NR2A KO mice showed higher levels of exploratory locomotion during initial exposure to a novel open field than wild-type controls (WT) ($n = 14–18/\text{genotype}$). (b) On average, NR2A KO mice spent more time in the center of the open field as compared to WT. ** $P < 0.01$, * $P < 0.05$ vs WT.

in Figure 5, NR2A KO, but not HET, mice showed significantly less immobility than WT controls in the forced swim test (main effect of genotype: $F_{2,43} = 4.02$; $P < 0.01$).

Antidepressant-Like Phenotype in the Tail Suspension Test

Genotype significantly affected behavior in the tail suspension test for depression-related activity. NR2A KO, but not HET, mice showed significantly less immobility than WT controls in the tail suspension test (main effect of genotype: $F_{2,42} = 17.91$; $P < 0.01$) (Figure 6).

Normal Physical Health, Sensory and Neurological Functions

As shown in Table 1, neither NR2A KO nor HET mice showed any gross behavioral abnormalities during exposure to an empty cage, with the exception that there was less defecation in both KO and HET mice than WT controls. All measures of physical health, and simple sensory and neurological functions were normal in NR2A KO and HET mice.

There were no genotype differences in the acoustic startle response (main effect of genotype: $P > 0.05$). There was a significant effect of prepulse intensity ($F_{2,78} = 90.68$; $P < 0.01$), but not genotype ($P > 0.05$) and no prepulse intensity \times genotype interaction ($P > 0.05$) on prepulse inhibition of the startle response. Data averaged across prepulse intensity are shown in Table 1.

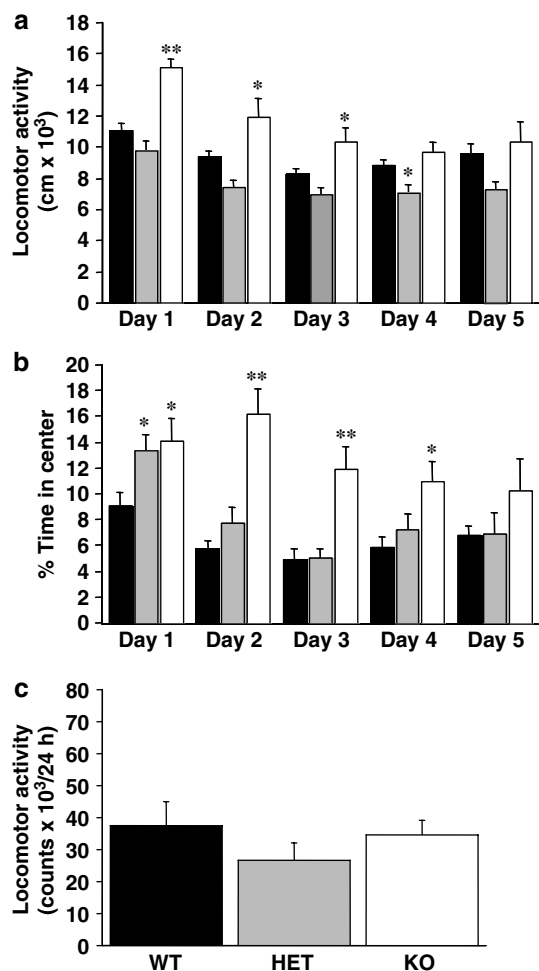


Figure 4 NR2A knockout (KO) mice show normal locomotor activity and reduced anxiety-like behavior in a familiar open field. (a) NR2A KO mice initially showed higher levels of locomotor activity in comparison to wild-type controls (WT), but do not differ from WT mice on days 4 and 5. Black bars = WT, grey bars = HET, white bars = KO ($n = 8-11$ /genotype). (b) Overall, NR2A KO mice spent more time in the center of the open field in comparison to WT controls. Black circles = WT, grey circles = HET, white circles = KO ($n = 8-11$ /genotype). (c) NR2A KO mice showed normal levels of locomotor activity in the non-aversive environment of the home cage ($n = 12$ /genotype). ** $P < 0.01$ vs WT, * $P < 0.05$ vs WT.

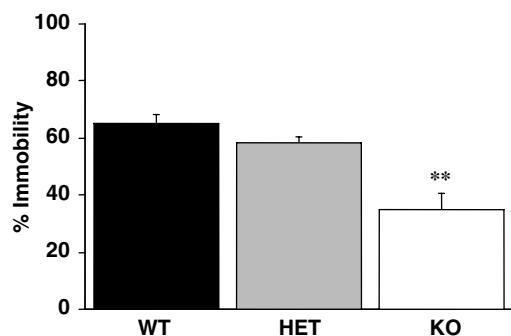


Figure 5 NR2A knockout (KO) mice exhibit reduced depression-related behavior in the forced swim test. NR2A KO mice showed less immobility than wild-type controls (WT) ($n = 13-18$ /genotype). ** $P < 0.01$ vs WT.

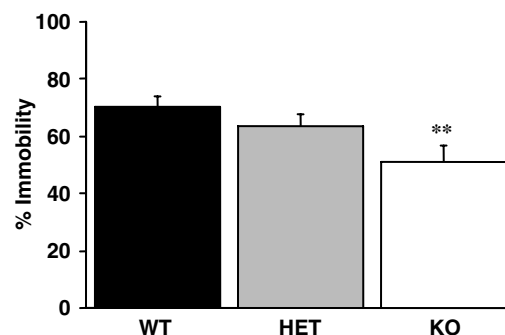


Figure 6 NR2A knockout (KO) mice exhibit reduced depression-related behavior in the tail suspension test. NR2A KO mice showed less immobility than wild-type controls (WT) ($n = 14-17$ /genotype). ** $P < 0.01$ vs WT.

DISCUSSION

The present study provides some of the first evidence linking the NR2A subunit of the NMDA receptor to the regulation of emotional behavior. NR2A KO mice exhibited profiles consistent with a reduction in anxiety-like behavior in the elevated plus-maze, light/dark exploration test and novel open field. In addition, NR2A KO mice displayed phenotypic alterations in the forced swim test and tail suspension test that were consistent with a reduction in depression-related behavior. NR2A HET mice showed similar albeit modest and nonsignificant changes in these tests, with the exception of the novel open field, where these mice showed a significant anxiolytic-like increase in center time, relative to WT controls. The phenotypic consistency observed in NR2A KO mice across tests is impressive given evidence that the tasks employed measure partially distinct forms of anxiety- and depression-related behavior, as evidenced in part by the previous studies showing that certain mutant mouse lines display phenotypic abnormalities on some of these tasks but not others (for reviews see (Cryan *et al*, 2005; Holmes, 2001)). Thus, present data demonstrate a highly robust anxiolytic- and antidepressant-like phenotype in NR2A KO mice.

Alterations in motor, sensory, and neurological functions resulting from a gene mutation can lead to false positives and can confound performance on mouse tests for anxiety and depression employed in the present study (Crawley, 2000; Holmes, 2001) (Holmes and Cryan, 2005). While NR2A KO mice exhibited greater levels of exploratory locomotor activity than WT controls in the novel open field, this difference occurred during the early part of the 60 min test, being especially prominent during the first 5 min and disappearing by the end of the test session. In a separate experiment, genotype differences in exploratory activity were abolished by repeated exposure to the open field, while NR2A KO mice continued to show greater center time than WT controls even when activity had habituated to WT levels.

Taken together, this pattern of behavior is suggestive of a reduced response to the anxiety-related component of the test rather than a nonspecific locomotor hyperactivity or an exaggerated response to novelty in the NR2A KO mouse; a finding consistent with data published previously (Miyamoto *et al*, 2001). Providing further support for this

Table 1 NR2A (knockout) KO Mice Show Normal Empty Cage Behaviors, Physical Health, Sensory Reflexes, and Neurological Functions

	WT	HET	KO
<i>Empty cage behaviors</i>			
Freezing	7	0	0
Trembling	0	0	0
Sniffing	100	100	100
Licking	0	0	0
Rearing	100	94	93
Jumping	0	0	0
Seizure	0	0	0
Defecation	71	22	14
Urination	7	11	14
Head bobbing	0	0	0
Circling	0	0	0
Abnormal gait	0	0	0
Retropulsion	0	0	0
<i>Physical health</i>			
Missing whiskers	0	0	0
Bald patches	0	0	0
Exophthalmus	0	0	0
Straub tail	0	0	0
Kinked tail	0	0	0
Kyphosis	0	0	0
Lordosis	0	0	0
Body weight (g)	23.4±0.9	22.2±0.7	20.8±0.9
Core body temperature (°C)	38.1±0.2	38.1±0.1	38.1±0.1
<i>Sensory reflexes</i>			
Approach response	100	100	100
Touch response	100	100	100
Palpebral response	100	100	100
Pinna reflex	100	100	100
Tail pinch response	100	100	100
Startle response (arbitrary units)	221±41	295±41	371±47
%Prepulse inhibition of startle	23.8±3.3	25.0±2.9	27.5±4.3
<i>Motor, neurological</i>			
Splayed limbs	0	0	0
Forepaw clutch	0	0	0
Hindpaw clutch	0	0	7

Fewer NR2A KO and HET mice defecated during exposure to the empty cage. Data denote the percentage of animals showing a response unless specified otherwise in parenthesis. % Prepulse inhibition of startle data is shown as an average over three prepulse intensities ($n = 12-18/\text{genotype}$).

interpretation, NR2A KO mice exhibited normal levels of locomotor activity in the non-aversive context of the home cage. In addition, NR2A KO mice were found to be no different from WT controls on a range of measures for basic motor abilities, physical health and sensory and neurological functions. Interestingly, NR2A KO and HET mice

defecated less than WT controls during brief exposure to an empty cage; providing another example of reduced emotional reactivity in these mice. Consistent with earlier findings, NR2A KO mice were also found to display normal prepulse inhibition of the acoustic startle response (Takeuchi *et al*, 2001). Thus, these data rule out the possibility that the presently observed emotion-related alterations in NR2A KO mice were an artifact of nonspecific behavioral or neurological abnormalities.

In contrast to these findings, an independently generated line of NR2A KO mice has been reported to show reductions in footshock-induced freezing, but normal behavior in the elevated plus-maze (although the only variable measured was open arm entries) (Moriya *et al*, 2000). Differences in procedural methodology as well as factors such as genetic background may have contributed to the differences between studies. Moriya *et al* (2000) studied NR2A KO mice on a 129/SvJ × C57BL/6 × DBA/2 × hybrid background, while a genome-wide scan of SNP markers confirmed that NR2A KO mice used in the present study were >99% C57BL/6J congenic. The present study also controlled for the potential influence of parental behaviors and early life experience (Holmes *et al*, 2005; Meaney, 2001) by breeding all genotypes from NR2A HET × NR2A HET parents. Thus, it may be that differences in these variables influenced the expression of an anxiety-related phenotype in the NR2A KO mouse across the two studies.

Notwithstanding, the finding that NR2A KO mice exhibit reduced anxiety- and depression-related behavior is consistent with previous pharmacological studies in rats showing anxiolytic- and antidepressant-like effects of competitive NMDA receptor antagonists and noncompetitive channel blockers in the same tasks as presently employed, including the elevated plus-maze, tail suspension test and forced swim test (Kelly *et al*, 1997; Papp and Moryl, 1994; Silvestre *et al*, 1997; Trullas and Skolnick, 1990; Wiley *et al*, 1995). In addition, systemic administration of zinc, one of the effects of which is inhibition of NR2A-subunit containing NMDA receptors, and treatment with glycine partial agonists have also been shown to produce antidepressant-like effects in these tests (Krocicka *et al*, 2001; Nowak *et al*, 2003; Trullas *et al*, 1989; Trullas and Skolnick, 1990; Zheng *et al*, 2001).

Taken together with present data, these results provide converging lines of evidence supporting anxiolytic- and antidepressant-like effects of inactivation of NR2A subunit containing NMDA receptors. However, the neural mechanisms underlying these effects remain to be determined. NR2A subunit-containing NMDA receptors are highly expressed in forebrain regions mediating emotion, such as the hippocampus and amygdala (Law *et al*, 2003; Magnusson *et al*, 2002; Sah and Lopez De Armentia, 2003). A simple model would predict that blockade of glutamatergic excitation of the neuronal activity in regions such as the amygdala would reduce emotional reactivity. However, emotional behavior is mediated by multiple neurotransmitter systems and the role of NMDA receptors in these behaviors is more likely to result from complex interactions with other systems (Paul and Skolnick, 2003). Of note in this context, NR2A KO mice have increased dopamine and serotonin metabolism in the frontal cortex, and decreased norepinephrine metabolism in the hippocampus (Miyamoto

et al, 2001). Although more direct measures of monoamine activity in these regions, such as extracellular fluid levels, have not been made, the possibility of altered monoamine function in NR2A KO mice is interesting given the long-standing hypothesis linking monoamines to emotional behavior (Charney, 1998).

Further studies are also needed to determine the precise nature of monoamine changes in NR2A KO mice and the possible contribution of these alterations to their anxiolytic- and antidepressant-like phenotype. Interestingly, however, previous studies have shown that NMDA receptor antagonists can block the antidepressant-like effects of monoamine-targeted drugs such as imipramine (Meloni *et al*, 1993). In addition, chronic treatment with monoaminergic antidepressants has been shown to antagonize and desensitize NMDA receptor function (Mjellem *et al*, 1993; Reynolds and Miller, 1988). Moreover, antidepressant effects of NMDA receptor antagonists, such as D-cycloserine, amantadine and ketamine have been reported in clinical populations (Berman *et al*, 2000; Crane, 1959; Vale *et al*, 1971). Thus, activity at NMDA receptors may contribute to the mechanism of action of the most commonly used pharmacotherapies for mood and anxiety disorders.

One implication of these findings, together with the present data, is that targeting NMDA receptors, and specifically the NR2A subunit, with selective antagonists could have efficacy as first-line or adjunct treatment for emotional disorders. Past experience has shown that the use of nonspecific NMDA receptor antagonists such as MK-801 and ketamine in neuropsychiatric disorders may be limited by their adverse side-effect profile (Javitt, 2004; Krystal *et al*, 1999). Blocking NMDA receptor function across brain regions would be predicted to cause general depression of the central nervous system (CNS), as well as produce specific impairments in cognition and possibly psychosis in certain at-risk individuals given the known role of the NMDA receptor in these behaviors (Goff and Coyle, 2001; Nakazawa *et al*, 2004). As the NR2A subunit clearly is part of a large population of mature NMDA receptors, it would seem unlikely that specifically targeting NR2A would circumvent all of these effects. Indeed, NR2A KO mice exhibit deficits in certain forms of synaptic plasticity and learning and memory performance, as well as hypersensitivity to behavioral effects of antipsychotics (Kishimoto *et al*, 2001; Kiyama *et al*, 1998; Miyamoto *et al*, 2001; Sakimura *et al*, 1995). There is also a caveat to basing the conclusion that targeting the NR2A will affect emotional behaviors solely on phenotypic data in the KO mouse. Inactivation of the NR2A subunit during development has the potential to indirectly cause abnormalities in NMDA receptor function, for example, *via* changes in other NR2 subunits, and these abnormalities could contribute to the emotional phenotype in NR2A KO mice. Of note in this context, however, there is no evidence of developmental alterations in the NR2B subunit in the NR2A KO mice (Townsend *et al*, 2003). Nor are there clear and consistent emotion-related effects of NR2B subunit antagonists in rodent tests (Dere *et al*, 2003; Fraser *et al*, 1996; Sanger and Jackson, 1989).

Notwithstanding, an important question for future studies will be to examine the anxiolytic- and antidepressant-like effects, as well the more general behavioral profile,

of drugs that can selectively block NR2A subunit function. These studies will also speak to the potential role of other NMDA receptor subunits in modulating emotional behavior, which is not ruled out by the present findings. To-date, however, the specificity of putative NR2A-selective antagonists remains somewhat controversial with little research of their antagonistic efficacy in brain following systemic treatment (Berberich *et al*, 2005; Liu *et al*, 2004a; Weitlauf *et al*, 2005).

In summary, the results of the present study demonstrate decreased anxiety- and depression-related behavior in NR2A KO mice on multiple tasks. This emotion-related phenotype was not due to more general deficits in locomotor activity or gross abnormalities in sensory or neurological functions. Anxiolytic- and antidepressant-like effects of NR2A gene inactivation are consistent with the effects of NMDA antagonists in rodent models and provide further insight into the molecular mechanisms underlying emotion and the pathophysiology and treatment of mood and anxiety disorders.

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