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GENETIC, PHARMACOLOGICAL AND LESION ANALYSES REVEAL A SELECTIVE ROLE FOR CORTICOHIPPOCAMPAL GLUN2B IN A NOVEL REPEATED SWIM STRESS PARADIGM

C. KISELYCZNYK^{a,b,*}, P. SVENNINGSSON^b, E. DELPIRE^c, and A. HOLMES^a

^aLaboratory for Behavioral and Genomic Neuroscience, National Institute on Alcohol Abuse and Alcoholism, Rockville, MD, USA

^bLaboratory of Translational Neuropharmacology, Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

^cDepartment of Anesthesiology, Vanderbilt University School of Medicine, Nashville, TN, USA

Abstract

Glutamate and N-methyl-D-aspartate receptor (NMDAR) dysfunction is strongly implicated in the pathophysiology of mood and anxiety disorders. Treatment with NMDAR antagonists has antidepressant efficacy in treatment-resistant depressives. In preclinical rodent models, NMDAR antagonist administration reduces anxiety- and stress-related behaviors in concert with increases in prefrontal cortical (PFC) dendritic spinogenesis and synaptic proteins. While these effects have been attributed to actions at the NMDAR GluN2B subunit, the precise role of cortical GluN2B in mediating emotional behaviors and stress-responsivity is not fully understood. Here, we employed a novel mutant model in which the GluN2B subunit is postnatally deleted in principal neurons in the cortex and the dorsal CA1 subregion of the hippocampus. GluN2BKO mice were phenotyped on a battery of tests for anxiety-related (light/dark exploration, stress-induced hyperthermia) and antidepressant-sensitive (sucrose preference, novelty-induced hypophagia, single-trial forced swim) behaviors. A novel repeated inescapable forced swim paradigm (riFS) was developed to assess behavioral responses to repeated stress in the GluN2BKO mice. For comparison, non-mutant C57BL/6J mice were tested for single-trial forced swim behavior after systemic Ro 25-6981 treatment and for riFS behavior after lesions of the ventromedial prefrontal cortex. riFS-induced alterations in corticolimbic GluN2B expression were also examined in C57BL/6J mice. We found that GluN2BKO mice reduced “despair-like” behavior in the riFS procedure, as compared to GluN2BFLOX controls. By contrast, GluN2BKO mice showed minimal alterations on anxiety-like or antidepressant-sensitive assays, including the single-trial forced swim test. In C57BL/6J mice, induction of “despair-like” responses in the riFS test was attenuated by vmPFC lesions, and was associated with changes in limbic GluN2B expression. Collectively, these data suggest that cortical GluN2B plays a major role in modulating adaptive responses to stress. Current findings provide further support for GluN2B as a key mechanism underlying stress

*Correspondence to: C. Kiselycznyk, Section on Behavioral Science and Genetics, Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, 5625 Fishers Lane, Room 2N09, Rockville, MD 20852-9411, USA. Tel: +1-301-443-4052; fax: +1-301-480-195. kiselycznykc@mail.nih.gov (C. Kiselycznyk).

responsivity, and a novel pharmacotherapeutic target for stress-related neuropsychiatric disorders.
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Keywords

glutamate; prefrontal cortex; amygdala; NMDA; anxiety; depression

There is intense interest in the contribution of glutamate dysfunction to the pathophysiology and treatment of mood and anxiety disorders (Conn and Jones, 2009; Skolnick et al., 2009). Glutamate is the major excitatory neurotransmitter in the brain and is a critical mediator of synaptic and behavioral plasticity. Glutamate binds to multiple metabotropic receptors and three families of ionotropic receptors: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), kainate receptors and N-methyl-D-aspartate receptors (NMDAR). Post mortem studies have found reduced NMDAR GluN1 subunit mRNA in the hippocampus of depressed patients (Law and Deakin, 2001) and decreased NMDAR GluN2A and GluN2B subunit protein levels in the prefrontal cortex (PFC) (Feyissa et al., 2009). These changes could reflect an adaptive response to NMDAR activation by elevated glutamate, given evidence from rodent studies showing that stress increases extracellular glutamate in these regions (Moghaddam, 1993).

Previous studies support the potential therapeutic value of targeting the NMDAR. In patients with major depression, a single treatment with the NMDAR antagonist ketamine has anxiolytic effects in healthy subjects (Krystal et al., 1994) and produces lasting antidepressant effects (aan het Rot et al., 2010; Berman et al., 2000; Zarate et al., 2006; Phelps et al., 2009; Price et al., 2009). In rats and mice, NMDAR antagonist treatment or loss of NMDAR gene function reduces anxiety- and depression-related behaviors on various tasks (Niewoehner et al., 2007; Halene et al., 2009; Barkus et al., 2010; Maeng et al., 2008; Li et al., 2010, 2011). NMDAR blockade also mitigates some of the behavioral and neural effects of stress. Systemic NMDAR antagonist treatment after exposure to chronic unpredictable stress prevents the expression of anhedonic- and anxiety-like behaviors and reverses loss of dendritic spines in the PFC (Li et al., 2011). NMDAR antagonists systemically administered concurrent with chronic restraint stress exposure prevents dendritic atrophy of rat hippocampal and PFC neurons (Magarinos and McEwen, 1995; Martin and Wellman, in press). Similarly, genetic inactivation of functional NMDARs on hippocampal CA3 pyramidal cells (via deletion of the obligatory GluN1 subunit) prevents stress-induced dendritic atrophy of CA3 and CA1 cells (Christian et al., 2011). However, this GluN1 deletion was not sufficient to block stress-induced changes in anxiety-like behavior, suggesting these behavioral effects stemmed from alterations in other regions, such as the PFC or amygdala.

NMDARs are heteromeric assemblies composed of an obligatory GluN1 subunit and one or more GluN2 (GluN2A-GluN2D) or GluN3 subunits (Rosenmund et al., 1998). GluN2A- and GluN2B-containing NMDARs are the predominant complexes in forebrain regions mediating emotional behaviors, such as the amygdala, ventral hippocampus and PFC. GluN2B-containing NMDARs are increasingly replaced by GluN2A-containing receptors

during postnatal development. This subunit switch results in a shorter duration NMDAR post-synaptic current (Flint et al., 1997; Lau and Zukin, 2007) and a decreased window for coincidence detection (Tang et al., 1999; Niewoehner et al., 2007; Halene et al., 2009) that may constrain excessive plasticity (Quinlan et al., 2004). The importance of subunit composition to the neuronal and plasticity-related properties of the NMDAR therefore raises the possibility that GluN2A and GluN2B may play differential roles in mediating stress and emotional behavior.

Preliminary support for this hypothesis comes from studies on mutant mice with NMDAR subunit-specific gene mutations. GluN2A knockout (KO) mice exhibit decreased anxiety- and depression-related behaviors at baseline and after chronic stress, and reduced basolateral amygdala (BLA) dendritic spine density (Boyce-Rustay and Holmes, 2006; Mozhui et al., 2010). Constitutive KO of GluN2B is lethal (Sprengel et al., 1998). However, GluN2B deletion restricted to pyramidal neurons throughout the forebrain, including hippocampus, amygdala and cortex, reduced anxiety-like behavior (von Engelhardt et al., 2008). These effects are recapitulated to some extent by acute systemic treatment with the GluN2B-selective antagonists ifenprodil and Ro 25-6981. Ifenprodil had an anxiolytic-like effect in one study (Fraser et al., 1996), while in other studies Ro 25-6981 did not affect anxiolytic-related behavior, but did produce an “antidepressant-like” effect in the forced swim test (FST) (Maeng et al., 2008; Li et al., 2010). Systemic Ro 25-6981 treatment has also been found to rescue stress-induced anhedonia and anxiety-like behavior in concert with increased PFC expression of spinogenesis-related synaptic proteins (Li et al., 2011). Supporting the translatable value of these preclinical data, initial clinical trials demonstrate antidepressant efficacy of the GluN2B-selective antagonist CP101,606 in treatment-resistant depressives (Preskorn et al., 2008).

The aim of the present study was to further clarify the role of GluN2B in anxiety- and stress-related behaviors using an integrated multi-technique approach. We employed a novel mutant model in which GluN2B is selectively, postnatally deleted in principal neurons throughout the cortex, as well as the dorsal CA1 subregion of the hippocampus (Brigman et al., 2010). These “GluN2B^{KO}” mice were phenotyped on a battery of tests for anxiety-related and antidepressant-sensitive behaviors. To assess GluN2B^{KO} mice for behavioral responses to repeated stress, we developed a novel repeated inescapable forced swim paradigm (riFS). To complement these phenotypic analyses, non-mutant C57BL/6J mice were tested for the effects of systemic Ro 25-6981 treatment and vmPFC lesions, and examined for riFS-induced *ex vivo* changes in corticolimbic GluN2B expression.

EXPERIMENTAL PROCEDURES

Subjects

GluN2B^{KO} mice were generated by crossing *Grin2b* floxed mutants with transgenic mice expressing Cre recombinase driven by the CaMKII promoter (T29-1 line) (Tsien et al., 1996) as previously described (Brigman et al., 2010). This intercross leads to restricted, postnatal deletion of GluN2B in principal cells of the cortex and dorsal CA1 hippocampus at the age-range tested here (i.e. 2–4 months). We have found that at later ages (~11 months of age) GluN2B deletion extends to striatum and ventral hippocampus (unpublished

observations). Mice were backcrossed to produce a >95% C57BL/6J background, as verified by genome scan (Brigman et al., 2010). GluN2B^{KO} and GluN2B^{FLOX} were littermates bred from GluN2B^{FLOX}/Cre-positive and GluN2B^{FLOX}/Cre-negative parents. Males and females were used and housed in same-sex groupings in a temperature- and humidity-controlled *vivarium* under a 12 h light/dark cycle (lights on 0600 h).

Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) at 2 months of age, housed two/cage and given at least 1 week of adaptation before testing. The number of mice used in all experiments is given in the figure legends. All testing was conducted during the light phase. Experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

Spontaneous anxiety- and depression-related behaviors in GluN2B^{KO} mice

We first phenotyped GluN2B^{KO} mice on a battery of tests that assay related, but non-overlapping, forms of spontaneous anxiety- and depression-related behavior. Putatively more stressful assays were conducted later in the sequence and there was at least 6 days interval between each assay. The order was (1) light-dark exploration, (2) stress-induced hyperthermia, (3) sucrose preference, (4) novelty-induced hypophagia, and (5) forced swim test/swim-induced corticosterone response.

Light-dark exploration

The light-dark exploration test is classic approach-avoidance conflict test for anxiety-related behavior (Crawley et al., 1981). The test was conducted as previously described (Mozhui et al., 2010). Mice began the test in an opaque black Plexiglas shelter (39×13×16 cm³) with a 1338 cm² opening at floor level that opened onto a large white Plexiglas square arena (39×39×35 cm³) illuminated to ~90 lux. Percent of time spent outside the shelter, frequency to exit the shelter, distance traveled outside the shelter, and the latency to first exit the shelter over a 15-min session were scored automatically by the Ethovision videotracking system (Noldus Information Technology Inc., Leesburg, VA, USA). Mice that did not exit the shelter were given a 900 s latency to exit. To dissociate the anxiety-related and general exploratory phases of the test session, data were separately analyzed during the first 5 min and last 5 min, respectively. Genotypes were compared by *t*-test.

Stress-induced hyperthermia

Stress-induced hyperthermia provides a simple locomotor-independent, physiological anxiety-related measure (Van der Heyden et al., 1997). The assay was conducted as previously described (Fitzgerald et al., 2010). Mice were individually housed in a clean, empty cage for 2 days to avoid disturbance resulting from handling of cage mates. Core body temperature was measured using a vegetable oil-lubricated thermometer probe (Thermalert TH-5, Physitemp, Clifton, NJ, USA) inserted 2 cm into the rectum for ~10 s, and the mouse was returned to the home cage. A second measurement was made 10 min later. Stress-induced hyperthermia was indicated by the delta increase in temperature between the two measurements and compared between genotypes by *t*-test.

Sucrose preference

Relative preference for a sucrose-sweetened solution (vs. water) provides a simple measure related to the anhedonia seen in depressive patients (Willner et al., 1987). Mice were individually housed for 2 days with *ad libitum* food and water, and then given free access to two bottles, one containing tap water and the other 1% sucrose solution, for 3 days. The left/right position of the bottles was alternated daily to avoid spurious effects from a side-bias. Fluid consumption was recorded each day (at 16:00 h), correcting for evaporation/spillage using an empty “dummy cage.” Genotypes were compared for sucrose preference (sucrose solution consumption/total fluid consumption averaged over the 3-day period) via *t*-test.

Novelty-induced hypophagia

The novelty-induced hypophagia test is a modification of the novelty-suppressed feeding test in which fluid or food consumption is inhibited by placing mice in a novel environment (Bodnoff et al., 1988). This test is sensitive to both anxiolytic and antidepressant drug treatments. The test was conducted as previously described (Karlsson et al., 2008). Mice were individually housed for 4 days with *ad libitum* food and water, and each day (at 13:00–16:00 h) presented for 15 min with diluted sweetened condensed milk (3:1 milk: water) in 50-ml Falcon tubes with a sipper top. The latency to lick and fluid consumed on the fourth day was taken as the “home cage” values. The following day, latency and consumption were measured in a clean, novel cage illuminated to 120 lux, with white paper placed underneath to further increase the aversive brightness. Mice were briefly placed in a transfer cage on both the novel cage (by necessity) and fourth home cage tests. The effects of genotype and cage-type were analyzed using 2-factor analysis of variance (ANOVA), with repeated measures for cage-type.

Forced swim and swim-induced corticosterone

Time spent immobile during forced swimming is a measure of “behavioral despair” that is sensitive to antidepressant drug treatment (Porsolt et al., 1978; Cryan and Holmes, 2005). The forced swim test was conducted as previously described (Karlsson et al., 2008). Mice were gently lowered into a 20-cm-diameter cylinder filled with 24 ± 1.0 °C water for a 6-min test. Immobility (cessation of limb movements except minor movement necessary to keep the mouse afloat) was scored every 5 s during min 3–6 and compared between genotypes using *t*-test.

To measure swim-induced serum corticosterone levels, mice were sacrificed 30 min after swim via cervical dislocation and decapitated as previously described (Boyce-Rustay et al., 2007). Trunk blood was collected, left to coagulate at room temperature for 1–2 h, then centrifuged at 4 °C for 30 s at 13,000 rpm. Serum was collected and analyzed using an MP Biomedicals Radioimmunoassay for Corticosterone (bound and free). Corticosterone levels were compared between genotypes using *t*-test.

Repeated inescapable forced swim (riFS) in C57BL/6J mice/effects of excitotoxic vmPFC lesions

Given the absence of phenotypic effects of restricted GluN2B deletion on measures of spontaneous anxiety- and depression-related measures, we next asked whether loss of the subunit affected adaptations to a repeated stressful experience. To this end, we used a modified version of a repeated forced swim test that was designed to induce “despair-like” immobility behavior, first by using a larger diameter cylinder (30 cm rather than 20 cm in standard version) and, second, the presentation, at the end of each trial, of a platform (plastic wiffle ball) under a 4×4 cm² escape hole 4 cm above the water line (see Fig. 2A). The platform was magnetically held 10-cm below the water until remotely released at the end of the trial. Once released, the platform quickly (<2 s) floated vertically upwards along a pole to sit atop the water directly under the escape hole. If the mouse attempted to climb onto or hold the platform, it sank below the water. The platform was presented after 1 min and trials terminated (typically within 20 s) regardless of attempts to use the platform. There was one trial per day from 10:00–12:00 h for 10 consecutive days. Immobility was scored every 5 s, as above, during the pre-platform period. To first determine the pattern of behavior across 10 riFS trials and the reliability of these changes across experiments, two separate cohorts of test-naïve non-mutant male C57BL/6J mice were tested as above. The effect of trial was analyzed using 1-factor ANOVA, with repeated measures for trial.

Next, given the largely cortical restriction of the GluN2B deletion in the mutants, and the known role of the vmPFC in mediating adaptations to stress (Maier et al., 2006; Holmes and Wellman, 2009) and putative prefrontal localization of the antidepressant-like effects of GluN2B antagonism (Li et al., 2010), we tested whether riFS behavior was sensitive to excitotoxic vmPFC lesions. Test-naïve C57BL/6J mice were anesthetized with isoflurane and the head was fixed into a stereotaxic device (Kopf Instruments, Tujunga, CA, USA). Injectors were bilaterally targeted at the infralimbic cortex based on coordinates (AP +2.00, ML ±0.30, DV –3.00 from bregma) from a mouse brain atlas (Paxinos and Franklin, 2001). Saline or 12.5 µg/ml NMDA (Sigma-Aldrich, St. Louis, MO, USA) was infused in a volume of 0.2 µl per hemisphere via 33-gauge infusion cannulas (Plastics One, Roanoke, VA, USA) attached with polyurethane tubing to a 2 µl Hamilton syringe (Hamilton, Reno, NV, USA). Solutions were slowly infused at a rate of 0.1 µl/min using a microinfusion pump (Harvard Apparatus PHD 22/2000, Holliston, MA, USA) and injectors remained in place for a further 5 min to allow complete diffusion into the tissue. Mice were then sutured and allowed to recover for 2 weeks prior to riFS testing as above. To histologically verify the location and extent of the lesions on completion of testing, mice were given an overdose of anesthetic, transcardially perfused with 4% paraformaldehyde and their brains removed. Coronal sections were cut on a vibratome to a thickness of 50 µm, plated on slides, and Nissl stained. Visual inspection of sections led to the exclusion of 6 of 19 subjects from the analysis.

Corticolimbic GluN2B and GluA1 protein expression after riFS

Next, we tested for changes in GluN2B protein levels in the vmPFC, dorsal hippocampus and BLA as a result of riFS exposure. This experiment also assessed whether riFS exposure affected “despair-related” behavior in the tail suspension test (TST) for antidepressant-related activity (Steru et al., 1985; Cryan and Holmes, 2005). C57BL/6J mice were

subjected to riFS as above, or left undisturbed in the home cage. The following day, mice were tested on the TST by suspension by the tip of the tail with tape to a wire cage lid suspended over a white Plexiglas square arena (39×39×35 cm³) that visually isolated each mouse, as previously described (Holmes et al., 2005). Thirty minutes later, mice were sacrificed via cervical dislocation and brain removal for freezing in ice-cold isopentane.

Bilateral samples from dorsal hippocampus, BLA and vmPFC were collected via tissue punch (2.0 μm for the dorsal hippocampus and vmPFC, 1.0 μm for BLA). Frozen tissue samples were sonicated in 1% SDS and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid protein assay method. Immunoblotting of GluN2B (antibody: Millipore cat# AB1557P) and GluA1 (antibody: Millipore cat# AB1504) was processed using 10% acrylamide gels, as previously described (Svenningsson et al., 2002). β-actin was used as a loading control. Antibody binding was detected by enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden) and quantified by densitometry using NIH IMAGE 1.61 software. Data were normalized to home cage control levels and groups compared using *t*-tests.

Systemic Ro 25-6981 treatment on riFS behavior in C57BL/6J mice

We next sought to investigate the role of GluN2B in the despair-like behavior in riFS with pharmacological experiments in non-mutant C57BL/6J mice. As we were mainly interested in the role of GluN2B in responses to repeated stress, we first tested for confounding acute behavioral effects of systemic GluN2B antagonism in the spontaneous forced swim and open field test. Mice were i.p. injected (10 ml/kg body weight) with 10 mg/kg Ro 25-6981 (Tocris, Ellisville, MO, USA) or 0.9% saline vehicle 30 min prior to the behavioral testing. This dose previously showed efficacy at reducing immobility in the forced swim (Maeng et al., 2008), and freezing in Pavlovian fear conditioning (Mathur et al., 2009). In the spontaneous forced swim, mice were tested as above and immobility scored every 5 s for the last 4 min of the test. In the open field test, mice were placed in a 40×40×35 cm³ square arena (60 lux) constructed of white Plexiglas for 30 min as previously described (Wiedholz et al., 2008). Total distance traveled in the whole arena and time spent in the center (20×20 cm²) was measured by the Ethovision videotracking system (Noldus Information Technology Inc., Leesburg, VA, USA). The effects of treatment and trial were analyzed using 2-factor ANOVA, with repeated measures for trial.

riFS in GluN2BKO mice

To examine the consequences of loss of GluN2B on behavior in the riFS test, a test-naïve cohort of GluN2B^{KO} mice was tested using the same procedure as described above. The effects of genotype and trial were analyzed using 2-factor ANOVA, with repeated measures for trial, followed by Bonferroni post hoc tests.

RESULTS

Normal spontaneous anxiety- and depression-related behaviors in GluN2BKO mice

Light-dark exploration—GluN2B^{KO} and GluN2B^{FLOX} mice did not differ in latency to exit the shelter (data not shown), number of exits from the shelter or time spent outside of

the shelter (Fig. 1A) during either the first or last 5 min of the test. Total distance traveled in the whole apparatus was significantly greater in GluN2B^{KO} than GluN2B^{FLOX} mice during the first ($t(38)=2.12$, $P<.05$) and last ($t(38)=3.194$, $P<.01$) 5 min of the test (Fig. 1B).

Stress-induced hyperthermia—Stress-induced delta increase in core body temperature ($\sim 1^\circ\text{C}$) was not different between GluN2B^{KO} and GluN2B^{FLOX} mice (Fig. 1C).

Sucrose preference—There was a strong preference for the sucrose-sweetened solution ($\sim 80\%$) that did not differ between GluN2B^{KO} and GluN2B^{FLOX} mice (Fig. 1D).

Novelty-induced hypophagia—There was a significant effect of cage environmental for latency to consume ($F(1,38)=55.43$, $P<.01$) and overall consumption ($F(1,38)=227.63$, $P<.01$) but no effect of genotype and no genotype $\times$ cage-type interaction for either measure. There was a longer latency to begin consuming (Fig. 1E) and lesser consumption overall (Fig. 1F) in the novel cage relative to the home cage, irrespective of genotype.

Forced swim and swim-induced corticosterone—GluN2B^{KO} and GluN2B^{FLOX} mice did not differ from each other in immobility during the forced swim test (Fig. 1G), or in serum corticosterone levels 30 min after swim (Fig. 1H).

riFS immobility in two separate cohorts of C57BL/6J mice—C57BL/6J mice showed vigorous escape-related activity during the first riFS trial and then a marked increase in immobility across subsequent trials ($F(9, 63)=10.67$, $P<.01$) (Fig. 2B). A similar pattern of behavior was seen in a replicate cohort ($F(9,36)=3.20$, $P<.01$) (Fig. 2C).

Decreased riFS immobility in vmPFC lesioned C57BL/6J mice—In lesioned mice, histological analysis showed that lesions encompassed the IL and prelimbic (PL) subregions of the vmPFC (Fig. 3A). There was a significant effect of lesion ($F(1,15)=4.86$; $P<.05$) and trial ($F(9,135)=15.53$, $P<.01$), but no trial $\times$ lesion interaction for immobility. Immobility increased across trials, regardless in both groups, but lesioned mice showed significantly less immobility than sham controls (Fig. 3B).

Altered GluN2B and GluA1 protein expression after riFS—There was a significant increase in immobility over riFS trials ($F(9,140)=6.59$, $P<.01$) (Fig. 4A). TST immobility was not different between riFS-exposed mice and home cage controls (Fig. 4B). To exclude the possibility that this negative result was an artifact of our TST procedure being poorly validated, we sought to confirm that the procedure was sensitive to antidepressant treatment (Porsolt et al., 1977). A naive cohort of C57BL/6J males were given an i.p. injection (10 ml/kg body weight) of 10 mg/kg fluoxetine (LKT Laboratories, Inc., St. Paul, MN, USA) or vehicle 30 min prior to TST testing. Results showed that fluoxetine treated mice were significantly less immobile than vehicle controls ($t(9)=3.358$; $P<0.01$) (data not shown).

In C57BL/6J mice exposed to riFS, GluN2B protein levels were significantly decreased in dorsal hippocampus ($t(12)=4.342$; $P<.01$) and significantly increased in BLA ($t(12)=2.455$; $P<.05$), but not significantly changed in vmPFC in riFS-exposed mice relative to home cage controls (Fig. 4C). GluA1 protein levels were also significantly decreased after riFS in

dorsal hippocampus ((12) $t=2.235$, $P<.05$) and showed a trend for an increase in BLA ((12) $t=1.666$, $P<.1215$), but were unaltered in vmPFC (Fig. 4D). β -actin levels were no different between riFS-exposed mice and controls for any blot.

Decreased riFS immobility after Ro 25-6981 treatment—In the spontaneous forced swim test, Ro 25-6981-treated mice showed a significant reduction in immobility after treatment ($t(10)=3.639$; $P<.01$) (Fig. 5A). An additional cohort displayed increased locomotion in the open field test after treatment ($F(1,10)=18.18$; $P<.01$) (Fig. 5B).

Decreased riFS immobility in GluN2B^{KO} mice—There was a significant effect of trial ($F(9,117)=11.51$, $P<.01$) and genotype ($F(1,13)=7.19$, $P<.05$) and no trial $\times$ genotype interaction ($F(9,117)=1.18$, $P<0.32$) for immobility. Immobility increased across trials, regardless of genotype. However, GluN2B^{KO} mice showed significantly less immobility than GluN2B^{FLOX} (Fig. 6).

DISCUSSION

The major finding of the current study was that genetic inactivation of GluN2B in principal neurons of the cortex (and hippocampal CA1) reduced “despair-like” behavior in response to repeated inescapable swim stress. By contrast, neither acute swim stress behavior, nor anxiety-like behaviors were affected by the mutation. In C57BL/6J mice, vmPFC lesions mimicked the GluN2B^{KO} behavioral response to repeated stress, and exposure to this stressor altered GluN2B (and GluA1) protein expression in amygdala and hippocampus.

A battery of tests measuring basal anxiety- and “depression-related” behaviors uncovered no phenotypic abnormalities in GluN2B^{KO} mice, as compared to GluN2B^{FLOX} controls. GluN2B^{KO} mice did show a modest increase in the total distance traveled by in the light/dark exploration task, suggestive of increased locomotor exploration. On the other hand, the mutants did not exhibit evidence of locomotor hyperactivity in the novelty-induced hypophagia or forced swim tests, where hyperactivity would have been predicted to manifest as reduced latency to drink and decreased immobility, respectively. Therefore there does not appear to be a strong locomotor hyperactivity in GluN2B^{KO} mice, a finding that provides an interesting contrast with the novelty-induced hyperactive phenotype produced by subunit-non-specific NMDAR mutation or antagonism (Mohn et al., 1999; Balu and Coyle, 2011).

The absence of an altered spontaneous anxiety- or “depression-related” phenotype in the GluN2B^{KO} mice differs from the decreased anxiety-like behavior recently reported in another model of GluN2B deletion tested on some of the same tests (black/white alley, light/dark exploration, modified elevated plus-maze) (von Engelhardt et al., 2008). Importantly, GluN2B deletion in the mice studies by von Engelhardt was more widespread than in the mutant we report here, encompassing much of the forebrain, including emotion-related regions such as ventral hippocampus and amygdala. Taking the two studies together suggests that widespread forebrain GluN2B deletion may be necessary to alter basal anxiety-like behavior, whereas loss of GluN2B restricted to cortex has more selective effects on behavioral adaptations to repeated stress (discussed further below). This scheme would be generally consistent with the majority of earlier work showing that lesions or NMDAR

antagonism in ventral hippocampus or amygdala decrease anxiety-like behavior, whereas lesions of cortex (or dorsal hippocampus) typically do not (Barkus et al., 2010; Nascimento Häckl and Carobrez, 2007; Holmes and Wellman, 2009). It will be interesting to clarify the role of GluN2B in specific brain regions using site-specific antagonism and gene knockdown in future studies.

To assess the behavioral response of GluN2B^{KO} mice to repeated stress, we employed a novel method: “riFS.” In this paradigm, mice are subjected to brief daily forced swims, each concluding with the presentation of a sinking “escape” platform. This method draws from a repeated swim procedure being pioneered by Stone and colleagues for rats and mice that induces long-term alterations to immobility behavior and is responsive to chronic, but not acute, antidepressants (Stone et al., 2008). We found that C57BL/6J mice engaged in vigorous swimming and escape attempts during early trials, but reliably developed increased immobility with repeated exposures. This “despair-like” response is reminiscent of the profile shown in a forced swim test by rats subjected to intermittent cold stress (Christianson and Drugan, 2005; Drugan et al., 2010). We found that although GluN2B^{KO} mice did develop a “despair-like” response in the riFS procedure, the magnitude of the response was significantly lower than that shown by GluN2B^{FLOX} controls. Importantly, GluN2B^{KO} mice tested on a standard, single exposure mouse version of the forced swim test were no different from GluN2B^{FLOX} controls, whereas, on the same test, C57BL/6J mice systemically treated with Ro 25-6981 showed reduced immobility. This latter observation concurs with prior studies in rat and mice (Maeng et al., 2008; Li et al., 2010), although interpretation of the effect as being genuinely “depression-related” was complicated by an open field locomotor hyperactivity effect at the same dose, that was not seen in prior work (Mathur et al., 2009; Haller et al., 2011). Notwithstanding, taking these findings together further supports a dissociation between an effect of brain-wide GluN2B inactivation on acute stress responsivity, and a more selective effect of loss of cortical/CA1 GluN2B impacting behavioral adaptations to repeated swim stress.

Recruitment of GluN2B under conditions of repeated stress would be in keeping with the importance of the subunit to synaptic and behavioral plasticity. Prior work has shown that transgenic GluN2B overexpression improves (Tang et al., 1999), while GluN2B deletion impairs (von Engelhardt et al., 2008; Brigman et al., 2010), various emotional (e.g. trace fear memory) and “non-emotional” (e.g. spatial memory) forms of learning and memory. Systemic treatment of rats and mice with Ro 25-6981 also impairs Pavlovian fear memory and extinction (Duffy et al., 2008; Haller et al., 2011; Mathur et al., 2009). Moreover, either GluN2B gene inactivation or Ro 25-6981 application disrupts long-term depression (LTD), and to some extent, long-term potentiating (LTP), at hippocampal synapses (Duffy et al., 2008; von Engelhardt et al., 2008; Brigman et al., 2010; Ge et al., 2011). Our findings add to this literature by demonstrating a role for GluN2B in mediating behavioral adaptations to repeated swim stress. Whether this stems from an enhancement of stress resilience or a failure to learn a particular response (of immobility) remains to be determined.

Another question current data cannot resolve is the relative contribution of loss of cortical versus CA1 hippocampal GluN2B to the stress phenotype in the mutants, because GluN2B is inactivated in both regions (Brigman et al., 2010). We did, however, find that excitotoxic

lesions of the vmPFC in non-mutant C57BL/6J mice mimicked the reduced “despair-like” riFS phenotype seen in the GluN2B^{KO} mice. Although we did not exclude the potential for a lesion-induced hyperlocomotor confound, we do not typically see hyperactivity after vmPFC lesions in our laboratory (Graybeal et al., unpublished observation), and nor do previous studies examining the effects of PFC lesions on anxiety- and stress-related behaviors (Bissiere et al., 2006; Jinks and McGregor, 1997; Shah and Treit, 2003). Rather, our lesion data are consistent with the importance of the vmPFC as a major target of stress and key regulator of behavioral responses to repeated stress (Maier et al., 2006; Holmes and Wellman, 2009). They also demonstrate that the riFS procedure is sensitive to vmPFC manipulations and thereby indirectly supports the contention that the GluN2B^{KO} phenotype is the result of loss of GluN2B in cortex rather than CA1.

Further support for this conclusion comes from recent elegant work by Li and colleagues (Li et al., 2010, 2011). Using the standard two-trial rat forced swim test, these authors recently found that systemic Ro 25-6981 treatment after an initial swim trial reduced immobility on a second trial, 24 h later (Li et al., 2010). These behavioral effects were associated with the induction of spinogenesis and plasticity-related protein expression in the PFC (Li et al., 2010). In a subsequent study, Li et al. have also found that a single systemic treatment with Ro 25-6981 reversed anhedonic and anxiety-like effects of chronic stress and prevented stress-induced loss of PFC synaptic proteins (Li et al., 2011). These findings suggest a mechanism by which cortical GluN2B might mediate adaptations to stress. In future studies, it would be valuable to test whether the attenuated “despair-like” response to repeated stress in the GluN2B^{KO} mice is accompanied by resistance to stress-induced molecular and structural changes in cortical neurons, as well as limbic target regions such as the amygdala. In this context, we found that riFS increased GluN2B expression in the BLA of C57BL/6J mice. This suggests that while the GluN2B in PFC may be the primary target of stress, a broader corticolimbic network of circuits is likely recruited to mediate stress adaptations.

In summary, the current study supports a role for GluN2B in mediating behavioral adaptations to stress. We show that genetically-induced loss of GluN2B in principal cells in the cortex and the CA1 region of the hippocampus reduced “despair-like” responses induced by repeated bouts of brief inescapable swim. The same mutation had minimal effects on basal anxiety-like or “depression-related” behaviors while, in non-mutant mice, the induction of “despair-like” responses altered limbic GluN2B expression and was behaviorally attenuated by vmPFC lesions. These findings provide further support for GluN2B as a key mechanism underlying stress responsivity, and a novel pharmacotherapeutic target for stress-related neuropsychiatric disorders.

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Abbreviations

BLA	basolateral amygdala
NMDAR	N-methyl-D-aspartate receptors
PFC	prefrontal cortex
riFS	repeated inescapable forced swim paradigm

Fig. 1. Normal spontaneous anxiety- and depression-related behaviors in GluN2B^{KO} mice. (A) Time spent outside of the shelter of the light/dark exploration test did not differ between GluN2B^{KO} and GluN2B^{FLOX} mice during the first or last 5 min of the test. (B) GluN2B^{KO} mice traveled significantly farther throughout the whole apparatus than GluN2B^{FLOX} controls during the first and last 5 min. (C) Stress-induced hyperthermia was no different between genotypes. (D) GluN2B^{KO} and GluN2B^{FLOX} mice showed similar sucrose preference. (E) Latency to drink milk was higher in a novel cage than a home cage, regardless of genotype. (F) Milk consumption was lesser in a novel cage than a home cage, irrespective of genotype. (G) Immobility in a standard, acute forced swim test was no different between GluN2B^{KO} and GluN2B^{FLOX} mice. (H) Corticosterone levels 30 min after forced swim were no different between genotypes. $n=18-22$ /genotype for behavioral tests, $n=6-7$ for corticosterone assay. Data are means $\pm$ SEM. * $P<.05$; ** $P<.01$; *** $P<.001$.

Fig. 2. Profile of repeated inescapable forced swim (riFS) immobility in C57BL/6J mice. (A) Diagram of the riFS test apparatus. An escape platform is held submerged below the surface of the water by a magnet on the outside of the tank. One minute into the trial, the platform is remotely released by switching off the magnet, allowing the platform to rise to the surface to sit beneath an escape hole but not be sufficiently buoyant to support the mouse. (B) Untreated C57BL/6J mice demonstrated a significant increase in immobility over 10 trials of riFS ($n=9$). (C) Replication of untreated C57BL/6J profile in an independent cohort ($n=5$). Data are means $\pm$ SEM.

Fig. 3. Reduced repeated inescapable forced swim (riFS) immobility in C57BL/6J mice with vmPFC lesions. (A) Schematic reconstruction of localization of excitotoxic lesions of the vmPFC. (B) vmPFC lesions significantly reduced riFS immobility in C57BL/6J mice relative to sham controls. $n=6-9/\text{group}$. Data are means $\pm$ SEM. * $P<0.05$.

Fig. 4.

Altered GluN2B and GluA1 protein expression in C57BL/6J mice after repeated inescapable forced swim (riFS). (A) Increased immobility across riFS trials in C57BL/6J mice. (B) Mice exposed to riFS did not differ from home cage controls in TST immobility. (C) GluN2B protein levels were decreased in dorsal hippocampus, increased in BLA, and unaltered in vmPFC in mice exposed to riFS, relative to home cage controls. (D) GluA1 protein levels were decreased in dorsal hippocampus, marginally increased in BLA, and unaltered in vmPFC, in mice exposed to riFS relative to home cage controls. $n=6-8/\text{group}$. Data are means $\pm$ SEM. * $P<0.05$.

Fig. 5.

Decreased repeated inescapable forced swim (riFS) immobility in C57BL/6J mice after Ro 25-6981 treatment. (A) Systemic injection of the GluN2B antagonist reduced immobility in the standard forced swim test ($n=6/\text{treatment}$). (B) Systemic GluN2B antagonist treatment increased total distance traveled in a novel open field test ($n=6/\text{treatment}$). Data are means $\pm$ SEM. ** $P < 0.01$.

Fig. 6.
Decreased repeated inescapable forced swim (riFS) immobility in GluN2BKO mice.
GluN2B^{KO} mice showed less immobility than GluN2B^{FLOX} controls across 10× daily riFS trials. $n=6-9$ /genotype. Data are means±SEM. * $P<0.05$.