GluN2D N-Methyl-D-Aspartate Receptor Subunit Contribution to the Stimulation of Brain Activity and Gamma Oscillations by Ketamine: Implications for Schizophrenia

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ABSTRACT
The dissociative anesthetic ketamine elicits symptoms of schizophrenia at subanesthetic doses by blocking N-methyl-D-aspartate receptors (NMDARs). This property led to a variety of studies resulting in the now well-supported theory that hypofunction of NMDARs is responsible for many of the symptoms of schizophrenia. However, the roles played by specific NMDAR subunits in different symptom components are unknown. To evaluate the potential contribution of GluN2D NMDAR subunits to antagonist-induced cortical activation and schizophrenia symptoms, we determined the ability of ketamine to alter regional brain activity and gamma frequency band neuronal oscillations in wild-type (WT) and GluN2D-knockout (GluN2D-KO) mice. In WT mice, ketamine (30 mg/kg, i.p.) significantly increased high-frequency oscillations in the medial prefrontal cortex (mPFC), entorhinal cortex and other brain regions, and decreased activity in the somatosensory cortex and inferior colliculus. In GluN2D-KO mice, however, ketamine did not significantly increase [14C]-2DG uptake in any brain region examined, yet still decreased [14C]-2DG uptake in the somatosensory cortex and inferior colliculus. Ketamine also increased locomotor activity in WT mice but not in GluN2D-KO mice. In electrocorticographic analysis, ketamine induced a 111% ± 16% increase in cortical gamma-band oscillatory power in WT mice, but only a 15% ± 12% increase in GluN2D-KO mice. Consistent with GluN2D involvement in schizophrenia-related neurologic changes, GluN2D-KO mice displayed impaired spatial memory acquisition and reduced parvalbumin (PV)-immunopositive staining compared with control mice. These results suggest a critical role of GluN2D-containing NMDARs in neuronal oscillations and ketamine’s psychotomimetic, dissociative effects and hence suggests a critical role for GluN2D subunits in cognition and perception.

Introduction

The discovery that the N-methyl-D-aspartate receptor (NMDAR) antagonists ketamine and phencyclidine (Anis et al., 1983) can mimic the symptoms of schizophrenia prompted genetic, biochemical, and pharmacologic studies resulting in the NMDAR-hypofunction theory of schizophrenia (Coyle et al., 2003; Lisman et al., 2008; Kantrowitz and Javitt, 2010). Pharmacologic blockade of NMDAR in healthy humans elicits a spectrum of schizophrenia symptoms, and NMDAR blockade in laboratory animals provides a model for schizophrenia (Kantrowitz and Javitt, 2010). Further support for the NMDAR hypofunction hypothesis comes from the identification of many schizophrenia candidate genes that impair NMDAR function (Sun et al., 2010; Balu and Coyle, 2011; Greenwood et al., 2012) and observations that decreasing NMDAR levels in mice through genetic manipulations leads to schizophrenia-associated symptoms (hyperlocomotor activity, impaired learning, reduced social interactions, and altered neuronal oscillations) (Mohn et al., 1999; Halene et al., 2009).

Precisely how NMDAR blockade induces schizophrenia symptoms is unclear, but many studies support the proposal that blockade of NMDARs in GABAergic interneurons containing parvalbumin (PV) is responsible for the psychotomimetic actions of NMDAR antagonists (Gonzalez-Burgos and Lewis, 2008; Lisman et al., 2008; Kantrowitz and Javitt, 2010). Because PV-interneurons provide negative feedback to pyramidal neurons, inhibition of NMDAR in PV cells causes an excitation of pyramidal neurons by disinhibition and thus alters the excitatory/inhibitory balance in cortical circuits (Li et al., 2011; Vautrot et al., 2012; Beer et al., 2014). The dissociative anesthetic ketamine elicits symptoms of schizophrenia at subanesthetic doses by blocking NMDARs. This property led to a variety of studies resulting in the now well-supported theory that hypofunction of NMDARs is responsible for many of the symptoms of schizophrenia. However, the roles played by specific NMDAR subunits in different symptom components are unknown. To evaluate the potential contribution of GluN2D NMDAR subunits to antagonist-induced cortical activation and schizophrenia symptoms, we determined the ability of ketamine to alter regional brain activity and gamma frequency band neuronal oscillations in wild-type (WT) and GluN2D-knockout (GluN2D-KO) mice. In WT mice, ketamine (30 mg/kg, i.p.) significantly increased high-frequency oscillations in the medial prefrontal cortex (mPFC), entorhinal cortex and other brain regions, and decreased activity in the somatosensory cortex and inferior colliculus. In GluN2D-KO mice, however, ketamine did not significantly increase [14C]-2DG uptake in any brain region examined, yet still decreased [14C]-2DG uptake in the somatosensory cortex and inferior colliculus. Ketamine also increased locomotor activity in WT mice but not in GluN2D-KO mice. In electrocorticographic analysis, ketamine induced a 111% ± 16% increase in cortical gamma-band oscillatory power in WT mice, but only a 15% ± 12% increase in GluN2D-KO mice. Consistent with GluN2D involvement in schizophrenia-related neurologic changes, GluN2D-KO mice displayed impaired spatial memory acquisition and reduced parvalbumin (PV)-immunopositive staining compared with control mice. These results suggest a critical role of GluN2D-containing NMDARs in neuronal oscillations and ketamine’s psychotomimetic, dissociative effects and hence suggests a critical role for GluN2D subunits in cognition and perception.

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et al., 2002; Homayoun and Moghaddam, 2007; Nakazawa et al., 2012). PV cell modulation also generates gamma frequency band neuronal network oscillations that are important for cortical processing, working memory, and perceptual integration (Sohal et al., 2009; Korotkova et al., 2010; Yizhar et al., 2011). Thus, acute administration of ketamine or phencyclidine (PCP) enhances excitatory activity in cortico-limbic structures and increases basal levels of gamma oscillations (Duncan et al., 1999; Duncan et al., 2000; Homayoun and Moghaddam, 2007; Nakazawa et al., 2012; Hunt and Kasiecki, 2013; Kocsis et al., 2013). Accordingly, selective reduction of the common GluN1 NMDAR subunit in PV cells, increases basal gamma oscillations, decreases NMDAR antagonist-induced gamma oscillations, and promotes schizophrenia-associated behavioral symptoms (Belforte et al., 2010; Korotkova et al., 2010; Carlen et al., 2012; Billingslea et al., 2014). These effects could also be mediated by PV-containing interneurons in the thalamic reticular nucleus (Frassoni et al., 1991; Llinas et al., 2005). In schizophrenia, NMDAR-hypofunction may thus disturb excitatory/inhibitory balance, thereby altering neuronal oscillations and disruptive cognitive function (Lisman et al., 2008; Kantzowitz and Javitt, 2010; Uhlhaas and Singer, 2013).

The roles played by NMDARs with different subunit combinations in cortical processing and schizophrenia-related symptoms are unknown. Such information is necessary for resolving individual pathophysiologic components of schizophrenia and for defining appropriate therapeutics. NMDARs are tetrameric complexes composed of two GluN1 subunits and two subunits from among the GluN2A-D and GluN3A-B subunits (Ikeda et al., 1992; Ishii et al., 1993; Mishina et al., 1993; Monyer et al., 1994; Traynelis et al., 2010). Pharmacologic studies in vivo have indicated a predominant role for GluN2A subunits in NMDAR antagonist-induced neuronal oscillations (Kocsis, 2012). However, in vitro experiments suggest a greater role for GluN2B subunits (McNally et al., 2011), and the role of GluN2C and GluN2D subunits is unclear. We hypothesized that GluN2D-containing NMDARs may contribute to ketamine-induced schizophrenia symptoms because GluN2D NMDAR subunits are localized in PV-containing GABAergic interneurons in cortex, reticular nucleus of thalamus, and hippocampus (Monyer et al., 1994; Standaert et al., 1996; Yamakawa et al., 2014; von Engelhardt et al., 2015) and because ketamine has a higher affinity for GluN2D-containing NMDARs than for NMDARs containing the more widely expressed GluN2A and GluN2B subunits (Watanabe et al., 1992; Watanabe et al., 1993b; Dravid et al., 2007; Kotermsani and Johnson, 2009). GluN2D involvement in schizophrenia could potentially also be mediated by altering neuronal-oligodendrocyte signaling (Fields, 2012; Micu et al., 2015). Thus, in the present study we sought to determine whether ketamine-induced cortical activation and gamma oscillations are reduced in GluN2D-KO mice.

The ketamine-induced increase in neuronal activity and gamma oscillations was determined by $^{[14]}$C-2DG uptake (reflecting neuronal activation) and by electrocorticography (ECoG) in wild-type (WT) and GluN2D knockout (GluN2D-KO) mice. In addition, if GluN2D subunits do contribute to schizophrenia symptoms, then drug-free GluN2D-KO mice may have behavioral defects and reduced PV expression as seen in schizophrenia patients (Lisman et al., 2008) and in rodents after chronic NMDAR blockade (Abekawa et al., 2007; Behrens et al., 2007; Benneyworth et al., 2011). Consequently, we also evaluated spatial learning and PV expression levels in untreated WT and GluN2D-KO mice. These findings demonstrate that GluN2D-containing NMDARs are necessary for full neuronal activation induced by ketamine and that GluN2D-hypofunction potentially contributes to schizophrenia symptoms.

Materials and Methods

Drugs. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Ketamine was purchased from Hospira (Lake Forest, IL), and $^{[14]}$C-2-deoxyglucose ($^{[14]}$C-2DG) was purchased from PerkinElmer (Boston, MA).

Animals. GluN2D-KO mice (Ikeda et al., 1995) that had been backcrossed onto a C57BL/6 background to 99.9% homogeneity (Hizue et al., 2005) were used for these studies. The background strain was confirmed to be congenic with C57BL/6 (Charles River Laboratories genetic testing service; Willington, MA). Mouse genotype was confirmed by polymerase chain reaction followed by sequencing of the reaction product and by Western blot analysis. Male C57BL/6 WT and GluN2D-KO mice 10 to 12 weeks of age were used for behavioral and 2DG uptake studies; 10- to 15-week-old male mice were used for ECoG studies. Mice were handled in accordance with University of Nebraska Medical Center’s Institutional Animal Care and Use Committee (IACUC) guidelines. In accordance with these guidelines, efforts were made to minimize animal suffering and the number of animals used.

2-Deoxyglucose Uptake Assay. Regional brain activity was determined by measuring $^{[14]}$C-2DG uptake (Kennedy et al., 1975) as previously described elsewhere (Kennedy et al., 1975; Duncan et al., 1999) with minimal modifications. Animals were injected with ketamine (30 mg/kg, i.p.) or saline and then injected after 2 minutes with $^{[14]}$C-2DG (0.16 μCi/μl). After another 15 minutes (i.e., 17 minutes after the ketamine/saline injection), the mice were decapitated under isoflurane anesthesia. Brains were isolated, rapidly frozen, and stored at −80°C. Horizontal brain sections (20 μm) were thaw-mounted onto glass slides and processed for autoradiography along with $^{[14]}$C-standards (ARC146; American Radiolabeled Chemicals, St. Louis, MO) using Kodak BioMax MR film (Carestream Health, Rochester, New York). Films were developed after 1 to 2 weeks of exposure and analyzed by quantitative image analysis (MCID system, St. Catharines, ON, Canada). Six to 8 brain sections were used for determining density for each brain region studied in each brain. Brain region absolute values were normalized by average radioactivity concentration of the whole corresponding section (Duncan et al., 1999).

Electrocorticography (ECoG). WT and GluN2D-KO mice were surgically implanted with tripolar electrodes (MS333/2; Plastics One, Roanoke, VA) under xylazine/ketamine/acepromazine anesthesia as required by IACUC regulations. Two holes were made in the skull 3 mm posterior to bregma at 1 mm and 2.5 mm lateral. Two electrodes were placed in the medial hole onto the dura surface near the retrosplenial cortex, and the third electrode was placed in the lateral hole for ground. The electrodes were secured to the skull as described elsewhere (Jeffrey et al., 2013). After 7 days of recovery, ECoG recordings were made with a DP-311 differential amplifier (Warner Instruments, Hamden, CT) with high-pass/low-pass filters set at 0.1 and 300 Hz and digitized/recorded (Digidata 1400, pClamp 10; Molecular Devices, Sunnyvale, CA). After 30 minutes of baseline recordings, the animals were injected i.p. with ketamine or saline and recorded for the period between 5 and 30 minutes after the injection. Ketamine administration (i.p.) in mice has an approximately 5-minute lag time and a peak ketamine response up to 30 to 45 minutes after injection (Phillips et al., 2012). In our initial studies, we found the peak response to be maintained through 20 minutes with a minor decrement by 30 minutes, so care was taken to match the recording periods between WT and GluN2D-KO mice. A power spectrum analysis was performed with Clampfit (Molecular Devices) using a Hamming window with 50% overlap.
In preliminary experiments, we found that the subanesthetic dose of 30 mg/kg i.p. gave a more robust augmentation of neuronal oscillations than 5 mg/kg. Ketamine is typically used in the 5–50 mg/kg range in electroencephalography experiments (Hunt and Kasicki, 2013).

**Open-Field Test.** For the open-field test (OFT), the floor of a plexiglas rectangular arena (40 × 30 cm) with 40 cm high walls was divided into 12 squares using black tape. Animals were treated with ketamine (30 mg/kg, i.p.) or saline, and their behavior was video recorded for 15 minutes. The arena was cleaned and rinsed with 70% ethanol between each animal. Video files were coded for blinded analysis of open-field line crosses (defined as both rear paws crossing over a line marked on the floor) and the number of entries into the two central squares. The number of rearings and wall-climbing attempts were also counted.

**Parvalbumin Immunohistochemistry.** Anesthetized mice were transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA)/PBS. Brains were removed and postfixed in 4% PFA for 24 hours at 4°C and then cryoprotected with 30% sucrose for another 24 hours. Brains were snap frozen and kept at −80°C until sectioned. Immunohistochemistry was performed in every fifth coronal cryostat section (40 μm) using the free-floating method. Sections were treated with 4% PFA for 15 minutes, rinsed (in PBS), and treated with 3% H2O2 for 30 minutes. After blocking with 10% bovine serum with 0.3% Triton X-100, sections were incubated with primary antibody (rabbit anti-PV, 1:10,000; Swant, Marl, Switzerland) for 48 hours at 4°C. Sections were washed and incubated with biotinylated secondary antibody (goat anti-rabbit, 1:200; Vector Laboratories, Burlingame, CA).

After washing, the sections were treated with ABC solution (Vector Laboratories), developed with 3,3′-diaminobenzidine, and coverslipped. Images were obtained by laser-scanning microscopy and PV-positive cells were counted using the National Institutes of Health ImageJ software.

**Spatial Memory in the Morris Water Maze.** The mice were given a 2-minute free-swim test the day before the start of training. Then for 3 sequential days, the mice were given four trials separated by 15 minutes on each day with a submerged platform located in the same position for all trials. In the training trials, the mice were allowed up to 1 minute to find the platform and were guided to the platform if they had not already found it. The mice then were allowed to stay on the platform for 15 seconds. The mice were placed in a different start location at the beginning of each trial with visual cues on the walls of the testing chamber. On day 4, the mice were tested with the platform removed (probe test). For each trial, we determined the time required to reach the platform. For each probe test, the percentage of time spent in the correct quadrant outside of the starting quadrant and the number of platform-site crossovers were determined.

**Statistical Analysis.** Prism 6 (GraphPad Software, San Diego, CA) was used for the statistical analysis. For most experiments, data were analyzed by two-way analysis of variance (ANOVA) with Sidak’s multiple comparison test to determine the difference among groups or Student’s t test as described elsewhere. P < 0.05 was considered statistically significant.

**Results**

**Effect of Ketamine on Regional Brain Activity as Demonstrated by 2DG Uptake.** Ketamine-induced regional changes in neuronal activation were measured by [14C]-2DG uptake quantitative autoradiography. Consistent with previous reports (Duncan et al., 1999; Miyamoto et al., 2000), ketamine (30 mg/kg) increased relative [14C]-2DG uptake (Fig. 1) in several brain regions and reduced uptake in others. [14C]-2DG uptake was quantified in 11 brain regions, and density differences were evaluated for statistical significance (Fig. 1B). Two-way ANOVA between regions and animal groups indicated an interaction effect [F (30, 314) = 6.00, P < 0.0001], a region effect [F (10, 314) = 33.6, P < 0.0001], and an animal group effect [F (3, 314) = 13.9, P < 0.0001]. In WT mice, ketamine increased relative [14C]-2DG uptake in the medial prefrontal cortex (mPFC, 37%, P < 0.0001), entorhinal cortex (34%, P = 0.0006), presubiculum (39%, P < 0.0001), and caudate putamen (21%, P = 0.018), and decreased relative uptake in the inferior colliculus (26%, P < 0.0001) and somatosensory cortex (23%, P = 0.0008) (Fig. 1B). Also, as others have reported (Duncan et al., 1999), for the whole section, absolute levels of [14C]-2DG uptake did not statistically significantly change with ketamine (WT/saline: 0.57 ± 0.06 nCi/mg tissue, n = 8; WT/ketamine: 0.52 ± 0.09, n = 9, P = 0.74; KO/saline: 0.40 ± 0.04 nCi/mg, n = 7; KO/ketamine 0.33 ± 0.04, n = 9, P = 0.74).

The distribution of [14C]-2DG uptake in GluN2D-KO mice after saline injection was similar to that seen in saline-treated WT mice (Fig. 1A) and was not statistically significantly different between genotypes in any brain region (Fig. 1B). In contrast to the WT mice, administration of ketamine did not cause a relative increase in [14C]-2DG uptake in any of the regions examined. Ketamine, however, decreased [14C]-2DG uptake in somatosensory cortex (15%, P = 0.0005), inferior colliculus (21%, P < 0.0001), and thalamus (13%, P = 0.0043).

**Ketamine Modulation of Neuronal Oscillations.** ECoG recordings of awake, stationary WT mice (n = 8) displayed a typical awake ECoG trace (Fig. 2A). Power spectrum analysis revealed that ketamine administration increased gamma frequency power (30–140 Hz) (Fig. 2, B and D) over baseline whereas ketamine in GluN2D-KO mice (n = 9) elicited a relatively small increase in power in the gamma range (and increased power between 140 and 170 Hz). As shown in Fig. 2D, the two genotypes appeared different between 60 Hz and 140 Hz, largely corresponding to high-frequency gamma oscillations as defined by Colgin et al. (2009) (65–140 Hz). Ketamine increased high gamma power more in WT mice (110.7% ± 16.4%) (Fig. 2E) than in GluN2D-KO mice (15.0% ± 11.6%, P = 0.0002, two-tailed t test). In GluN2D-KO mice, ketamine treatment was associated with a peak of variable magnitude near 155 Hz; in ketamine-treated WT mice, there was a peak near 135 Hz (Fig. 2D), also of variable magnitude but of consistent peak frequency.

**Ketamine-Induced Motor Activity.** As measured in the OFT, ketamine (30 mg/kg, i.p.) increased locomotor activity in WT mice during the 15 minutes after injection (Fig. 3, A and B). In the WT mice, the average number of squares crossed after ketamine treatment was statistically significantly greater (528.0 ± 62.3, n = 8) than after saline treatment (264.0 ± 43.4, n = 7, P = 0.0005). Ketamine did not statistically significantly induce hyperlocomotion in GluN2D-KO mice (squares crossed in the saline condition: 171.4 ± 20.0, n = 7; ketamine: 222.7 ± 31.6, n = 10; P = 0.64). The two genotypes were different in the ketamine condition (P < 0.0001) but not in the saline condition (P = 0.31).

In the OFT, avoidance of the central piece of the central squares was nearly doubled in ketamine-treated mice than in the saline treated group (Fig. 3C). Two-way ANOVA indicated statistically significant effects of
genotype \((P < 0.0001)\) and treatment \((P = 0.0005)\), and multiple comparisons testing (Sidak’s) indicated a difference between saline and ketamine in the WT \((P = 0.0006)\) but not in the GluN2D-KO mice \((P = 0.35)\). Thus, ketamine failed to significantly increase entries into the central squares by GluN2D-KO mice in parallel with effects on locomotor activity.

In contrast to the blunting effect that eliminating GluN2D had on ketamine-induced hyperlocomotion, ketamine fully reduced rearings and climbing attempts in both WT and GluN2D-KO mice (Fig. 3, D and E). However, in the saline controls, GluN2D-KO mice had a statistically significantly lower level of rearings and climbing attempts than WT mice \((P < 0.0001, P = 0.019)\), respectively, Sidak’s multicomparison test). The stereotypical behavior of walking in circles (rotations) was induced by ketamine administration in both genotypes (Fig. 3F), but was statistically significantly greater \((P = 0.0008, \text{Sidak’s multicomparison test})\) in WT \((56.4 \pm 12.8, n = 9)\) than in GluN2D-KO mice \((14.4 \pm 3.0, n = 10)\).

**Parvalbumin Immunohistochemistry.** The inability of the psychotomimetic agent ketamine to increase activation of the prefrontal cortex and increase basal gamma oscillations in GluN2D-KO mice suggests that GluN2D-containing NMDARs contribute to psychotomimetic activity. If GluN2D subunit activity contributes to the defects seen with NMDAR hypofunction, which is associated with a decreased expression of PV in the schizophrenic brain and in animal models after chronic NMDAR blockade (Abekawa et al., 2007), then the expression of PV may be reduced in the GluN2D-KO mouse. Two-way ANOVA of PV cell staining indicated an interaction effect \([F (5,36) = 2.865, P = 0.028]\), a genotype effect \([F (1,36) = 19.5, P < 0.0001]\), and a region effect \([F (5,36) = 124.1, P < 0.0001]\) with multicomparison testing indicating a statistically significantly lower density of PV-positive cells in the GluN2D-KO substantia nigra \((P = 0.0038)\) and in the basolateral/lateral amygdala \((P = 0.0051)\) compared with WT mice (Fig. 4). PV expression levels were also lower in mPFC and hippocampus in the GluN2D-KO, but these decreases were not statistically significant.
Evaluation of Disruption in Spatial Memory and Sensory-Motor Gating. If elimination of GluN2D subunits reproduces some aspects of schizophrenia-related NMDAR hypofunction, then GluN2D-KO mice may have defects in spatial memory acquisition, a function disturbed in schizophrenia and related to cortical neuronal oscillations. During spatial learning task acquisition, WT mice appeared to show greater improvement from trial to trial than did GluN2D-KO mice (Fig. 5A). Two-way ANOVA analysis indicated a statistically significant effect of trial \( [F(11,216) = 6.85, P < 0.0001] \) and genotype \( [F(11,216) = 7.95, P = 0.0053] \) on the observed variation, with no statistically significant interaction \( [F(11,216) = 0.68, P = 0.76] \) and no statistically significant difference for any of the 12 individual training trials. However, following the 12th training trial, removal of the platform and measurement of the percentage of time spent in the correct quadrant of the time spent outside of the starting quadrant revealed statistically significantly better performance (more time in the correct quadrant) by the WT mice than the GluN2D-KO mice (WT: 56.0% ± 6.0%, n = 10; KO: 34.6% ± 3.5%, n = 10; \( P = 0.0063 \), two-tailed \( t \) test) (Fig. 5B). Similarly, WT mice crossed the former position of the removed platform a greater number of times than did the KO mice (WT: 4.1 ± 0.7, n = 10; KO: 1.5 ± 0.5, n = 10; \( P = 0.010 \), two-tailed \( t \) test) (Fig. 5C). The average swimming speed was not different between WT and KO mice. Thus, GluN2D-KO mice have impaired learning of the spatial memory task compared with WT mice.

Results from the sensory-motor gating experiments (pre-pulse inhibition) are shown in the supplemental material (Supplemental Fig. 1).

Discussion

Evidence from a variety of genetic, biochemical, and pharmacologic studies support the concept that NMDAR hypofunction contributes to many of the diverse symptoms of schizophrenia (Coyle, 2006; Lisman et al., 2008; Kantrowitz and Javitt, 2010). However, the relationships between specific NMDAR subtypes and schizophrenia symptom components are not well understood. Psychotomimetic agents, such as ketamine and PCP, produce many of the symptoms of schizophrenia by modulating the neuronal systems known to underlie schizophrenia (for reviews, see Lahti et al., 1995; Javitt, 2007; Lodge and Mercier, 2015). Hence, resolving how these agents act on specific NMDAR subtypes to produce psychotomimetic symptoms should help define mechanisms of drug action as well as neurobiologic mechanisms underlying schizophrenia.

In this study, we found that GluN2D-KO mice had a greatly reduced activation of brain activity in response to ketamine. Using \(^{14}\text{C}\)-2DG uptake to reflect regional brain metabolic activity and the excitatory/inhibitory balance, we found that the characteristic increase in corticolimbic activation seen in rodents (Duncan et al., 1999) and humans (Vollenweider et al.,...
after ketamine administration was not seen in GluN2D-KO mice. This finding is consistent with the very recent report that ketamine-induced nitric oxide synthase activation is dependent upon GluN2D subunits (Yamamoto et al., 2015). Because GluN2D subunits are found in PV-containing GABAergic interneurons of cortex, hippocampus, and thalamus (Standaert et al., 1996; Yamasaki et al., 2014; von Engelhardt et al., 2015), these results support the hypothesis that ketamine causes corticolimbic activation by inhibiting PV interneurons and thereby disinhibiting excitatory neurons.

Interestingly, ketamine was still able to decrease 2DG uptake in somatosensory cortex and inferior colliculus in GluN2D-KO mice as it does in WT mice (Fig. 1). In contrast to these effects of GluN2D deletion, a global reduction in GluN1 subunits blunts both the NMDAR antagonist-induced increase in 2DG uptake in corticolimbic regions as well as the antagonist-induced decrease in other brain regions (Duncan et al., 2002). Thus, GluN2D subunits contribute to excitatory disinhibition, an important subset of ketamine’s actions, but not to other effects of ketamine. The ketamine-induced reduction in 2DG uptake in somatosensory cortex that persists in the GluN2D-KO potentially reflects the ketamine blockade of GluN2C-containing receptors in thalamic reticular nucleus interneurons, which would promote delta/theta oscillations and reduced activity in somatosensory cortex via the specific thalamocortical projections (Llinas et al., 2005; Zhang et al., 2009).

Our finding that GluN2D deletion reduces ketamine-induced gamma oscillations suggests that GluN2D-containing NMDARs have an important role in modulating neuronal network oscillations. This has significant implications for schizophrenia. Neuronal oscillations in the gamma frequency band are thought to be integral to cognition and perception, and their impairment has been proposed to underlie the symptoms of schizophrenia (Gonzalez-Burgos and Lewis, 2008; Gonzalez-Burgos et al., 2010; Uhlhaas and Singer, 2013). Because NMDARs in PV cells are important for the modulation of gamma oscillations (Carlen et al., 2012; Uhlhaas and Singer, 2013), these results are also consistent with a key role of GluN2D subunits in cortical PV cell function. In addition, GluN2D subunits in the thalamus are likely to contribute to ketamine-induced dysrhythmias. Nucleus reuniens participates in circuits involved in schizophrenia-related symptoms (prefrontal cortex, hippocampus, and ventral tegmentum) (Lisman et al., 2010; Duan et al., 2015; Griffin, 2015; Ito et al., 2015) and is enriched in GluN2D subunits (Watanabe et al., 1993a; Buller et al., 1994). Additionally, inhibition of NMDAR in the reticular nucleus, which contains GluN2D and GluN2C subunits (Watanabe et al., 1993a; Yamasaki et al., 2014), generates telencephalic delta oscillations and potentially schizophrenia-related symptoms (Zhang et al., 2009).
Thus, GluN2D subunit-containing NMDARs may have an essential role in the pathophysiologic expression of NMDAR hypofunction that underlies schizophrenia’s cognitive symptoms. This suggestion is consistent with studies associating genetic variants of GluN2D subunits with schizophrenia risk (Makino et al., 2005) and with reduced GluN2D expression in schizophrenia (Sodhi et al., 2011) and in an animal model of schizophrenia (Bullock et al., 2009).

Ketamine also produced an increase in oscillations at frequencies corresponding to high-frequency oscillations (HFO), as previously reported elsewhere (Hunt and Kasicki, 2013). In GluN2D mice, the ketamine-induced peak appeared at a higher frequency (~155 Hz) than in WT mice (~135 Hz). Interestingly, other groups have reported a similar finding in the methylazoxymethanol acetate neurodevelopmental model of schizophrenia. Hagino et al. (2010) have shown that the enhanced release of dopamine and hyperlocomotor activity occurring after PCP administration is absent in GluN2D mice, so GluN2D may be contributing to the positive symptoms of schizophrenia (Hagino et al., 2010). These findings are supported by the reduction in ketamine-induced hyperlocomotor and rotation activity in GluN2D-KO mice (Yamamoto et al., 2015). This action of PCP/ketamine may possibly be due to the presence of GluN2D subunits in the ventral tegmental area/substantia nigra, basal ganglia, and/or in the midline thalamic nuclei (Beaton et al., 1992; Watanabe et al., 1992; Buller et al., 1994; Monyer et al., 1994).

Other GluN2 NMDAR subunits also are likely to be involved in schizophrenia pathophysiology. Genetic studies show a strong association of schizophrenia with both GluN2A and GluN2B subunit genes (Allen et al., 2008; Greenwood et al., 2012). GluN2A subunits have also been implicated by pharmacologic studies of gamma oscillation modulation in vivo.
PV levels in interneurons of the basolateral amygdala and performance in a spatial memory task (Fig. 5). The impact of nigra reticulata and amygdala (Fig. 4) and a reduced performance in schizophrenia or after chronic NMDAR blockade. Consistently, one might expect similar defects in the adult GluN2D-KO mouse as seen during development (Wang et al., 2001) are associated with an altered excitatory/inhibitory balance, reduced PV expression, and disturbed gamma oscillation modulation (Lisman et al., 2008; Kantrowitz and Javitt, 2010; Uhlhaas and Singer, 2013). If GluN2D-containing NMDARs contribute to the excitatory/inhibitory balance during development, one might expect similar defects in the adult GluN2D-KO mouse as seen in schizophrenia or after chronic NMDAR blockade. Consistent with this possibility, untreated GluN2D-KO mice are associated with a reduced expression of PV in the substantia nigra reticulata and amygdala (Fig. 4) and a reduced performance in a spatial memory task (Fig. 5). The impact of PV levels in interneurons of the basolateral amygdala and substantia nigra on oscillations is not yet known. However, if the trend in reduced PV expression in other brain regions (Fig. 4) is meaningful, these changes could potentially contribute to facilitated gamma oscillations and behavioral deficits (Vreugdenhil et al., 2003; Wohr et al., 2015).

GluN2D-KO mice may thus model some but not all components of schizophrenia. Reduction in prepulse inhibition (PPI) is thought to be a sensitive measure in schizophrenia. However, GluN2D-KO mice have a robust PPI response (Takeuchi et al., 2001), a result confirmed in our laboratory (Supplemental Fig. 1). Elimination of the PPI response appears to require actions at multiple NMDARs. Knocking out or knocking down individual GluN2 subunits does not reduce PPI (Takeuchi et al., 2001; Spooren et al., 2004), but combining pharmacologic inhibition of GluN2B-containing receptors in the GluN2A KO mouse (Spooren et al., 2004) or knocking down GluN1 subunits globally (Frady et al., 2005) does reduce PPI. Reduction in PPI was also not seen after GluN1 ablation from PV cells (Korotkova et al., 2010), thus the NMDAR-associated neural substrate for impaired PPI function appears distinct from the system modulating gamma oscillations through NMDARs on PV cells. Our results are thus consistent with the report that low doses of ketamine increase rather than decrease PPI in humans (Abel et al., 2003).

In summary, the inability of the psychotomimetic agent ketamine to: 1) increase metabolic activation in corticolimbic regions, 2) increase basal gamma oscillations, 3) increase locomotor activity, and 4) increase stereotypical rotations in GluN2D-KO mice suggests that GluN2D-containing NMDARs contribute to the psychotomimetic activity of ketamine. We also find that GluN2D elimination through development results in a partial down-regulation of PV similar to that seen in schizophrenia and after chronic NMDAR blockade. Together, these results suggest that GluN2D subunits might contribute significantly to the neuronal networks thought to be pivotal in cognitive processing, which are disrupted in schizophrenia. These findings suggest that pharmacologic augmentation of signaling mediated by GluN2D-containing NMDARs may be of therapeutic benefit in schizophrenia, a finding consistent with recent animal studies (Suryavanshi et al., 2014).

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References

Fig. 5. Spatial memory acquisition in WT and GluN2D-KO mice. Spatial memory was tested in the Morris Water Maze with the time necessary to first reach the submerged platform (latency) measured for (A) each successive trial (four per day, 3 successive days). On the fourth day, the submerged platform was removed and in the subsequent test trial, (B) the percentage of time spent in the correct quadrant outside of the starting quadrant was measured, as was (C) the number of times the mouse passed over the prior location of the removed platform. Both test measures of task acquisition were statistically significant between WT and GluN2D-KO (n = 10 for each group, *P < 0.05; two-tailed t test).

(McNally et al., 2011; Koesis, 2012) and PV down-regulation (Kinney et al., 2006). However, GluN2B-selective antagonists were found to better augment kainate-induced gamma oscillations than a GluN2A-prefering antagonist (McNally et al., 2011). GluN2C involvement in schizophrenia is suggested by the finding that GluN2C transcript levels are significantly reduced in schizophrenia patients (Weickert et al., 2013) and by the presence of working memory and fear acquisition deficits in GluN2C-KO mice (Hillman et al., 2011). A potential role of GluN2C subunits in schizophrenia is also suggested by GluN2C expression in the reticular nucleus of the thalamus, which modulates hippocampal delta oscillations and thus may explain the delta oscillatory changes seen in schizophrenia patients (Zhang et al., 2012).

Schizophrenia and chronic blockade of NMDARs during development (Wang et al., 2001) are associated with an altered excitatory/inhibitory balance, reduced PV expression, and disturbed gamma oscillation modulation (Lisman et al., 2008; Kantrowitz and Javitt, 2010; Uhlhaas and Singer, 2013). If GluN2D-containing NMDARs contribute to the excitatory/inhibitory balance during development, one might expect similar defects in the adult GluN2D-KO mouse as seen in schizophrenia or after chronic NMDAR blockade. Consistent with this possibility, untreated GluN2D-KO mice are associated with a reduced expression of PV in the substantia nigra reticulata and amygdala (Fig. 4) and a reduced performance in a spatial memory task (Fig. 5). The impact of PV levels in interneurons of the basolateral amygdala and substantia nigra on oscillations is not yet known. However, if the trend in reduced PV expression in other brain regions (Fig. 4) is meaningful, these changes could potentially contribute to facilitated gamma oscillations and behavioral deficits (Vreugdenhil et al., 2003; Wohr et al., 2015).

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