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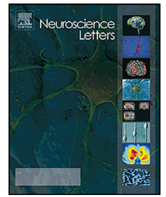

Research paper

Role of the NMDA receptor GluN2D subunit in the expression of ketamine-induced behavioral sensitization and region-specific activation of neuronal nitric oxide synthase

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HIGHLIGHTS

- GluN2D subunit is essential for ketamine-induced behavioral sensitization.
- Ketamine rapidly increases neuronal NOS activity in the striatum and frontal cortex.
- Fronto-striatal pathway is important for ketamine-induced behavioral sensitization.

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ABSTRACT

The present study aimed to investigate the involvement of the NMDA receptor (NMDAR) and/or nitric oxide (NO) pathway in ketamine-induced behavioral sensitization. Mice received repeated subcutaneous administration of ketamine (25 mg/kg), once daily or once weekly for a total of five doses. Even three administrations of ketamine, daily or weekly, induced a rapid increase in locomotor activity in wild-type (WT), but not in GluN2D knockout (GluN2D-KO) mice. Furthermore, for WT mice receiving daily ketamine, elevated locomotor activity was maintained after a 1-month withdrawal period; however, this was not the case when ketamine was administered weekly.

The effect of acute ketamine on nNOS activities was estimated with nicotinamide adenine dinucleotide hydrogen phosphate-diaphorase (NADPH-d) histochemistry. Ketamine rapidly increased the number of NADPH-d activated cells and strongly stained dendrites in the dorsal striatum and prefrontal cortex of WT mice, but not GluN2D-KO mice. These results suggest that ketamine-induced locomotor sensitization and nNOS activation in the frontal cortex–striatum neuronal circuit are positively correlated and that the NMDAR GluN2D subunit plays an important role in the acquisition and maintenance of ketamine-induced behavioral sensitization.

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1. Introduction

Ketamine is a dissociative anesthetic and is well known to be a noncompetitive antagonist of the *N*-methyl-D-aspartate (NMDA) receptor. Ketamine is psychotomimetic and is used recreationally as a rave drug. Research has shown that administration of ketamine to rodents at weekly intervals results in the development of locomotor sensitization [20], which indicates a risk of ketamine abuse in humans.

The NMDA receptor (NMDAR) ion channel consists of two GluN1 subunits and two GluN2 (A–D) or GluN3 (A–B) subunits. At a physiological Mg^{2+} concentration (1 mM), ketamine preferentially

Abbreviations: CPP, 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid; GluN2D-KO, GluN2D knockout; NADPH-d, nicotinamide adenine dinucleotide hydrogen phosphate diaphorase; NBT, nitroblue tetrazolium; NMDAR, NMDA receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PB, phosphate buffer; PBS, phosphate buffered saline; PCP, phencyclidine; WT, wild type.

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inhibits GluN2C- and/or GluN2D-containing NMDARs [16]. NMDAR inhibition increases glutamate release in the prefrontal cortex and causes cortical excitation by disinhibition of pyramidal neurons [17]. Glutamatergic neurons in the frontal cortex innervate the striatum. Stimulation of NMDARs increases intracellular Ca^{2+} and activates neuronal nitric oxide synthase (nNOS) in the postsynaptic neuron. Therefore, NMDARs are functionally coupled to nNOS, which regulates nitric oxide (NO) production via Ca^{2+}-calmodulin binding [2,3,5]. Several studies have shown that the neuronal NO system is involved in behavioral sensitization induced by psychostimulants, such as cocaine or methamphetamine [4,14]. nNOS knockout (KO) mice fail to develop long-lasting sensitization to cocaine, similar to mice in which nNOS inhibitors are used to block nNOS [12,18]. Furthermore, NO is essential for methamphetamine sensitization [1,13]. NADPH-d (nicotinamide adenine dinucleotide hydrogen phosphate diaphorase) histochemical staining can be used as a reliable marker to estimate the distribution of nNOS enzyme activity in both cell bodies and dendritic processes [7,11].

Ketamine is psychoactive and has been used to develop a pharmacological model for human schizophrenia. Therefore, investigation of the molecular mechanisms of the psychomimetic properties of ketamine is important for the development of fast-acting antidepressants without side-effects. The present study was undertaken to assess the role of the NMDAR GluN2D subunit and NO synthesis on the development of ketamine-induced behavioral sensitization. Here we demonstrate that GluN2D-KO mice fail to develop ketamine-induced locomotor sensitization and nNOS activation.

2. Materials and methods

2.1. Drugs and reagents

Ketamine hydrochloride, nitroblue tetrazolium (NBT), and Triton X-100 were purchased from Sigma–Aldrich (St. Louis, MO, USA), and β-NADPH tetrasodium salt was purchased from Roche Diagnostics (Indianapolis, IN, USA).

2.2. Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Yokohama City University and conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Male C57BL/6J (CREA Japan, 25–35 g) and GluN2D-KO mice and wild-type littermates (WT) (25–35 g) were used in the present study. Generation of the mutant mice lacking the GluN2D NMDAR subunit has been described elsewhere [15]. All mice were housed under standard laboratory conditions for a 12-h/12-h light/dark cycle. Food and water were available *ad libitum.*

2.3. Drug treatment

Ketamine hydrochloride was dissolved in saline and administered in single doses at 5–25 mg/kg, subcutaneously (s.c.). Behavioral sensitization was assessed by subchronic treatment with ketamine (25 mg/kg, s.c., once daily or weekly) for a total of five doses. After a 1-month withdrawal period, the sustainability of ketamine-induced behavioral sensitization was examined with an additional single dose of ketamine (25 mg/kg, s.c.). Control animals received saline.

2.4. Spontaneous locomotor activity

Locomotor activity was measured with an automated video tracking system (Animal Vision System, RS0013; RENET, Tokyo, Japan). The position of the animal was determined every 100 ms. Mice were placed in the locomotor activity arena (54 cm diameter × 40 cm high) and allowed to habituate for 60 min before administration of saline or ketamine. Locomotor data were collected in 5-min bins.

Dose-response studies were performed with male C57BL/6J mice to determine the optimal ketamine dosage for the locomotor activity experiment. Mice received an injection of saline or ketamine at 5, 10, or 25 mg/kg, s.c. ($N = 5$ per dose).

To induce behavioral sensitization, WT and GluN2D-KO mouse received once daily or once weekly injections of ketamine (25 mg/kg, s.c.) for a total of five doses. Immediately after every injection, the mouse was placed into the center of the open field and allowed to roam freely in the arena.

2.5. NADPH-diaphorase histochemistry

Ten minutes after ketamine injection (0, 10, 25 or 50 mg/kg, s.c.), mice were deeply anesthetized with diethyl ether and transcardially perfused with 0.1 M phosphate buffered saline (PBS), followed by fixation with 0.4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and post-fixed for 2 h in 0.4% paraformaldehyde in 0.1 M PB. The fixed brains were incubated overnight in 20% sucrose in 0.1 M PB at 4 °C and were then serially cut in the coronal plane at 40 μm thickness on a vibratome (VT 1000S, Leica Microsystems, Nussloch, Germany). NADPH diaphorase (NADPH-d) histochemical staining was then performed on the brain sections to determine the localization of neurons with nNOS activation, according to the method described by Dawson et al. [6] with some additional optimization. The free-floating sections were permeabilized with 0.3% Triton X-100 in 0.1 M PBS for 1 h at room temperature and then incubated with 0.5 mg/ml β-NADPH, 0.2 mg/ml NBT, and 0.3% Triton X-100 in 0.1 M Tris-HCl, pH 7.4, at 37 °C for 40 min. The sections were mounted onto slides and air dried, dehydrated, and coverslipped with Entellan® (Merck KGaA, Darmstadt, Germany).

Digital images were captured with a Keyence microscope (BD-9000, Keyence, Osaka, Japan). NADPH-d reactive neurons were detected by the presence of the blue tetrazolium reaction product in cell bodies and processes.

Quantification of NADPH-d reactive neurons was performed for five serial sections from each brain. The number of stained neurons was counted with image processing software (Scion Image). The sections examined were located caudal to the nucleus accumbens (NAc; Bregma 1.42–1.22 mm), according to the Franklin and Paxinos mouse brain atlas [9].

2.6. Statistical analysis

All experimental data are presented as mean ± SEM. Statistical significance was determined with an analysis of variance (ANOVA) and Student's *t*-test in GraphPad® Prism 5.0 software. One-way and two-way ANOVA with Bonferroni post-hoc tests was used to determine if there were significant differences between groups assayed at different times following different treatments. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Acute ketamine administration induced dose-dependent increases in locomotor activity

Subcutaneous ketamine administration was accompanied by a marked increase in locomotor activity in C57BL/6J mice. The onset of hyperactivity occurred immediately after injection and peaked during the first 10 min after injection, followed by a return

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