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Differential expression of NMDA receptors in serotonergic and/or GABAergic neurons in the midbrain periaqueductal gray of the mouse

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HIGHLIGHTS

- Whole cell recordings and real time RT-PCR were applied to neurons in the PAG.
- Non-serotonergic/GABAergic neurons exhibited a high NMDA/non-NMDA ratio of EPSC.
- Serotonergic neurons exhibited a low NMDA/non-NMDA ratio of EPSC.
- Peripheral nerve ligation upregulated NR2B in non-serotonergic/non-GABAergic neurons.
- Differential regulation of NMDAR in the PAG seems involved in neuropathic pain.

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptors expressed in the midbrain periaqueductal gray (PAG) exert various physiological functions. The PAG contains various neurotransmitter phenotypes, which include GABAergic neurons and serotonergic neurons. In the present experiments, we made tight-seal whole-cell recordings from GABAergic and/or serotonergic neurons in mouse PAG slices and analyzed NMDA and non-NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) evoked by electrical stimulation. The NMDA/non-NMDA ratio of EPSC amplitude was high and the decay time course of NMDA-EPSC was slow in non-serotonergic/GABAergic neurons. In contrast, serotonergic neurons exhibited a low NMDA/non-NMDA ratio and a fast decay time course of NMDA-EPSC. Peripheral nerve ligation-induced chronic pain was associated with an increased NMDA/non-NMDA ratio in serotonergic neurons. Additionally, single-cell real-time RT-PCR analysis showed that peripheral nerve ligation up-regulated NR2B subunit expression in non-serotonergic/non-GABAergic neurons. Such changes in NMDA receptor expression in the PAG result in an alteration of the descending modulation of nociception, which might be an underlying mechanism for peripheral nerve injury-evoked persistent pain. Finally, the expression of NMDA receptors seems differentially regulated among neurons of different neurotransmitter phenotypes in the PAG.

1. Introduction

The midbrain periaqueductal gray (PAG) is involved in various physiological functions, which include nociceptive modulation, emotion and anxiety, autonomic regulation and so on [2]. Regarding nociceptive modulation, the PAG, in company with the rostral ventromedial medulla, constitutes a major component of the descending pain control system [8,14,28].

Autoradiographic and *in situ* hybridization studies have shown that *N*-methyl-D-aspartate (NMDA) receptors are distributed in the PAG [1,25]. With regard to physiological roles of NMDA receptors in the PAG, opposing facilitatory and inhibitory modulation of nociceptive transmission have been described. Some reports suggested that NMDA receptors in the PAG exert an inhibitory effect on nociceptive response [4,6,15]. In contrast, other reports suggested that NMDA receptors in the PAG are critically involved in hyperalgesic pain states induced by peripheral injury or inflammation [10,11,27]. Furthermore, it has been reported that persistent pain induced by complete Freund's adjuvant (CFA) injection or by

Abbreviations: PAG, periaqueductal gray; NMDA, *N*-methyl-D-aspartate; GABAγ, -aminobutyric acid; EPSC, excitatory postsynaptic current; RT, reverse transcription; PCR, polymerase chain reaction; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; APV, 2-amino-5-phosphonovalerate; CFA, complete Freund's adjuvant; NSE, neuron specific enolase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GAD67, glutamate decarboxylase 67; TPH2, tryptophan hydroxylase 2.

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chronic constrictive nerve injury is associated with up-regulation of the NMDA receptor mRNA in the PAG, suggesting the pathophysiological roles of NMDA receptors in chronic pain [12,18].

Neurons in the PAG are heterogeneous, containing different neurotransitters such as γ-amino-butyric acid (GABA), serotonin and glutamate [3,22]. These neurons are implicated in various physiological functions depending on their neurotransmitters. Additionally, NMDA receptors expressed in PAG neurons with different neurotransmitter phenotypes may play different physiological roles.

In the present experiments on mouse PAG slices, by means of tight-seal whole-cell recordings combined with single-cell real-time reverse transcription-polymerase chain reaction (RT-PCR), we analyzed the NMDA receptor-mediated synaptic transmission in GABAergic and/or serotonergic neurons and the effects of partial ligation of the sciatic nerve.

2. Materials and methods

2.1. Animals

Male ICR mice 6- to 9-weeks-old were used for the present experiments. The care and use of the animals were in accordance with institutional guidelines and the guidelines of the International Association for the Study of Pain [29].

2.2. Surgery

Mice were anesthetized with halothane for the nerve-ligation surgery and for the sham surgery. The left sciatic nerve was partially ligated according to the protocol described by Seltzer et al. [21]. In the sham surgery, the sciatic nerve was exposed, but not ligated.

2.3. Assessment of mechanical hypersensitivity

The mechanical sensitivity of the hindpaw was assessed by measuring the frequency of withdrawal of the foot to mechanical stimuli. Stimuli were applied to the plantar surface of the foot with a von Frey filament (0.5 g). Each trial consisted of the application of a von Frey filament ten times. The occurrence of foot withdrawal for each trial was expressed as a percentage response frequency. The measurements were made from 2 days before the surgery through 14 days after the surgery.

2.4. Preparation of midbrain slices

The mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and decapitated. The brains were quickly removed and blocked. Transverse slices (350 μm) were cut by a vibratome (DTK-1500, Dosaka, Japan). Slices were transferred to a recording chamber, which was continually superfused (2–3 ml/min) with an artificial cerebral spinal fluid of the following composition (mM): NaCl, 113; KCl, 3; $NaHCO_3$, 25; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; D-glucose, 11, equilibrated with 95%O_2–5%CO_2. When necessary, $MgCl_2$ was excluded to make a nominally Mg^{2+}-free solution.

2.5. Tight-seal whole-cell recordings

Tight-seal whole-cell recordings were made from cells visually localized in the PAG. Patch pipettes were filled with an internal solution having the following ionic composition (mM): potassium gluconate, 123; KCl, 14; sodium gluconate, 2; EGTA, 1; HEPES, 10; pH neutralized to 7.4 with KOH. For perforated patch-clamp recordings, amphotericin B (6 μg/ml) was included in the pipette solution. All experiments were performed at room temperature.

Excitatory postsynaptic current (EPSC) was evoked at 0.1 Hz using a stimulating electrode filled with 1 M NaCl with tip diameter of *ca.* 3 μm placed about 200 μm away from the recording site. All recordings were made in the presence of strychnine (1 μM), and bicuculline (10 μM). Non-NMDA-EPSC was recorded in the presence of a NMDA receptor antagonist, 2-amino-5-phosphonovalerate (APV, 50 μM) at holding potential of −70 mV. NMDA-EPSC was recorded in the presence of a non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) in the nominally Mg^{2+}-free solution at a holding potential of −70 mV. EPSCs were recorded in the voltage clamp mode using an Axopatch 200B patch clamp amplifier (Axon Instruments, USA). Data were sampled at a rate of 10.0 kHz through a Digidata 1440A (Axon Instruments).

2.6. Single-cell real-time RT-PCR

After whole-cell recordings were made, the neurons were aspirated into another pipette according to a previously described protocol [26]. The neurons were then ejected into thin-walled autoclaved PCR tubes by applying a gentle positive pressure, and immediately frozen and stored at −80 °C until use. On the following day, lysis was performed using IGEPAL CA-630 at room temperature for 5 min followed by RT with Superscript II (Life Technologies, USA).

After the RT reaction, preamplification of cDNA samples was performed using the TaqMan PreAmp Master Mix (Applied Biosystems, USA) according to the manufacturer's directions using the recommended program for 14 cycles. Then preamplified cDNA was utilized for real-time PCR using TaqMan Fast Universal Master Mix and TaqMan Gene Expression Assays (Applied Biosystems). The reaction was performed on an Eppendorf Real Plex 2 (Eppendorf, Germany). Samples were assayed in triplicate. Each particular gene was regarded as present in individual single cell samples when its fluorescence intensity exceeded a predetermined threshold value in <45 cycles of PCR.

TaqMan gene expression assays were performed for the following genes: neuron specific enolase (NSE, Assay ID: Mm00469062_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1), glutamate decarboxylase-67 (GAD67, Mm00725661_s1), tryptophan hydroxylase-2 (TPH2, Mm00557715_m1), NMDA receptor NR1 subunit (NR1, Mm00433790_m1), and NMDA receptor NR2B subunit (NR2B, Mm00433820_m1). GAPDH served as a positive control and NSE served as a neuronal marker.

2.7. Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were carried out using a two-way analysis of variance (ANOVA) followed by a simple effects test and by post hoc multiple comparisons using Tukey's test. The χ^2 test with Yates' correction was also used when appropriate. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. NMDA-EPSC and non-NMDA-EPSC in serotonergic and/or GABAergic neurons

A graph in Fig. 1A illustrates an example of single-cell real-time RT-PCR amplification plots of six mRNAs in a PAG neuron. The expression of both GAD67 and TPH2 mRNAs indicates that the neuron under observation was serotonergic and GABAergic. Electrical stimulation of nearby passing fibers evoked fast EPSC in the presence of 2 mM Mg^{2+} and 50 μM APV (blue trace in the lower