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Peripheral AMPA receptors contribute to muscle nociception and *c-fos* activation

Yang-Hyun Chun [b], Dorie Frank [a], Jong-Seok Lee [a], Youping Zhang [a], Q-Schick Auh [b], Jin Y. Ro [a,*]

[a] *Department of Biomedical Sciences, Program in Neuroscience, University of Maryland, Baltimore, School of Dentistry, 650 W, Baltimore Street Baltimore, MD 21201, USA*
[b] *Kyung Hee University, School of Dentistry, Department of Oral Medicine, 1 Hoegi Dong, Dongdaemun Gu, Seoul, Republic of Korea*

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Abstract

In this study, involvement of peripheral AMPA receptors in mediating craniofacial muscle pain was investigated. AMPA receptor subunits, GluR1 and GluR2, were predominantly expressed in small to medium size neurons but more GluR2 positive labeling were encountered in trigeminal ganglia (TG) of male Sprague Dawley rats. A greater prevalence of GluR2 is reflected by the significantly higher percentage of GluR2 than GluR1 positive masseter afferents. Nocifensive behavior and *c-fos* immunoreactivity were assessed from the same animals that received intramuscular mustard oil (MO) with or without NBQX, a potent AMPA/KA receptor antagonist. Masseteric MO produced nocifensive hindpaw shaking responses that peaked in the first 30 s and gradually diminished over a few minutes. There was a significant difference in both peak and overall MO-induced nocifensive responses between NBQX and vehicle pre-treated rats. Subsequent Fos studies also showed that peripheral NBQX pre-treatment effectively reduced the MO-induced neuronal activation in the subnucleus caudalis of the trigeminal nerve (Vc). These combined results provide compelling evidence that acute muscle nociception is mediated, in part, by peripherally located AMPA/KA receptors, and that blockade of multiple peripheral glutamate receptor subtypes may provide a more effective means of reducing muscular pain and central neuronal activation.

Keywords: Muscle pain; Mustard oil; Peripheral glutamate receptors; Masseter

1. Introduction

Glutamate receptors located on peripheral terminals of nociceptors are activated by glutamate released from various sources such as neurons, Schwan cells, and macrophages, following injury or inflammation (Piani et al., 1991; Parpura et al., 1995; deGroot et al., 2000; Lawand et al., 2000). Glutamate administered directly to the masseter muscle activates and sensitizes nociceptors in rats and produces pain in human subjects via peripheral NMDA receptors (Cairns et al., 2003, 2006). Blockade of peripheral NMDA receptors significantly reduces mustard oil (MO)- and hypertonic saline-induced nocifensive behaviors, edema formation in the masseter, and *c-fos* activation in the caudal subnucleus of the spinal trigeminal nucleus (Vc), a major nociceptive processing area for orofacial input (Ro, 2003; Ro et al., 2004, 2007).

Similarly, local administration of an mGluR5, but not mGluR1, antagonist effectively reduces the MO-induced nocifensive behavior and plasma extravasation in the rat masseter (Lee et al., 2006). Consistent with this observation, 3,5-dihydroxyphenylglycol (DHPG), a group I mGluR agonist, mediated masseter hypersensitivity is significantly attenuated when an mGluR5 antagonist is co-administered (Lee and Ro, 2007a). These data strongly suggest that peripheral mGluR and NMDA receptors are involved in localized muscle pain, mechanical hypersensitivity and inflammation.

While it is likely that AMPA/KA receptors could also contribute to muscle nociception, relative involvement of peripheral AMPA/KA receptors in muscle nociception are yet to be elucidated. This is an important gap to fill since peripherally released glutamate will obviously activate all subtypes of glutamate receptors. This study investigates whether AMPA

* Corresponding author. Tel.: +1 410 706 6027; fax: +1 410 706 4172.
E-mail address: jro@umaryland.edu (J.Y. Ro).

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receptor subunits are expressed specifically in masseter afferents, and blockade of peripheral AMPA/KA receptors significantly reduces MO-induced nocifensive behaviors and *c-fos* activation in the Vc.

2. Materials and methods

2.1. Animals and general procedure

Experiments were performed on male Sprague Dawley rats (250–350 g) housed in a temperature-controlled room under a 12:12 light–dark cycle with access to food and water ad libitum. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23) and under a University of Maryland approved Institutional Animal Care and Use Committee protocol.

2.2. Immunohistochemistry for AMPA receptor subunits on masseter afferents

Fast Blue (FB) (2%; 10 μl) was injected in the masseter bilaterally to retrogradely label muscle afferents in trigeminal ganglia (TG) in two rats. After 7 days to allow the FB to label masseter afferents animals were perfused transcardially with phosphate buffer solution (PBS) followed by 4% paraformaldehyde in PBS (250 ml; pH 7.2). TG was extracted and post-fixed for 90 min, placed in 10% sucrose solution at 4 °C overnight, then sectioned coronally at 10–15 μm. The sections were incubated overnight with primary antisera for GluR1 or GluR2 (both Chemicon; 1:250). For immunofluorescence the sections were incubated at 37 °C for 30 min with Cy-3 conjugated goat anti-rabbit antiserum (West Grove, PA; 1:250). The primary antibody was omitted from the processing of selected sections to control for nonspecific background staining. GluR1/2 positive cells were counted from representative sections obtained from four TG. Labeled TG neurons were classified as small (>400 μm^2), medium (400–1000 μm^2), and large cells (<1000 μm^2) (Ichikawa and Sugimoto, 2001). The soma sizes were measured from labeled neurons that showed clear nucleolus. Percentages of masseter afferents double labeled with GluR1 or GluR2 were compared with the *t*-test. The significance of this and all subsequent statistical analyses was set at $p < 0.05$.

2.3. Behavioral study and data analysis

Rats were assigned to one of the following groups. Four groups of rats ($n = 8$ each) were pretreated with either a potent AMPA/KA antagonist, NBQX disodium salt, (1, 50 and 100 nmol/10 μl; Tocris Cookson), or vehicle control (PBS) in the masseter 5 min prior to MO (20% in 30 μl) injection in the same muscle. Another group of rats ($n = 8$) received the highest dose of NBQX (100 nmol) in the forelimb muscle 5 min prior to MO injection in the masseter to control for possible systemic effects of NBQX.

We have previously shown that algesic stimulation of the masseter muscle evokes ipsilateral hindpaw shaking responses in lightly anesthetized rats (Ro et al., 2003). Using the same behavioral paradigm we quantified the MO-induced hindpaw shaking responses in NBQX or vehicle treated rats by counting the number of shakes in 30-s intervals. The counts were plotted over time to determine the temporal pattern of response. The highest number of shakes occurring during the first 30-s block was taken as the peak count (PC). The area under the curve (AUC) for each plot was obtained as a measure of overall magnitude of response. All counts were made by one experimenter blinded to experimental conditions to maintain the consistency of counting. Mean PC (MPC) and AUC were analyzed using one-way analysis of variance (ANOVA). Dunnett's test was used for post-hoc comparisons. The data are presented as mean ± SEM.

2.4. c-fos study and data analysis

In order to correlate the behavioral responses with Fos-like immunoreactivity (Fos-LI) in the Vc, the same animals used in the behavioral study were also used for the Fos immunohistochemistry study. We maintained the following subset of animals used in the behavioral study and collected their brainstem tissue for subsequent processing of Fos-LI: NBQX with MO (1 nmol, $n = 6$; 50 nmol, $n = 7$; 100 nmol, $n = 7$), vehicle with MO ($n = 6$), and NBQX (forelimb) with MO (100 nmol, $n = 6$). No additional post-injection experimental manipulation was performed on these animals to ensure that Fos data was not influenced by any procedural variables.

After 2 h following documentation of behavioral responses, these animals received a lethal dose of sodium pentobarbital (100 mg/kg, i.v.) followed by transcardiac perfusion with 4% paraformaldehyde in PBS (pH 7.3). The lower brainstem (from the obex to 5-mm caudal to the obex) was blocked and post-fixed overnight. Blocks were serially sectioned (30 μm) and every fourth section was processed Fos-LI. Free floating sections were incubated successively in 5% normal donkey serum (30 min), affinity-purified rabbit polyclonal anti-Fos antibody (Oncogene Science; 1:20,000; overnight), biotinylated donkey anti-rabbit antibody (Chemicon International; 1:300; 1 h), and avidin-biotin-peroxidase complex (Vector Laboratory; 1 h). Diaminobenzidine was used for visualization of Fos-LI. Primary antibody was omitted from processing of selected sections to serve as a control for nonspecific staining.

The Vc was referenced to the obex according to coordinates provided by Paxinos and Watson (1998). For the purpose of this study, we analyzed Fos-LI in the caudal Vc by counting Fos positive cells in 10 representative tissue sections taken from 3 to 5 mm caudal from the obex. The average number of Fos positive cells was obtained for each rat by dividing the total number of Fos positive cells by 10 and used for statistical comparisons between the groups with one-way ANOVA and Dunnett's post-hoc test. All counts were made by one experimenter to maintain the consistency in application of criteria used to select profiles as Fos positive cells and reduce the likelihood for subject variability. The experimenter was blinded to experimental conditions.

3. Results

3.1. AMPA receptors subunits are expressed in TG muscle afferents

GluR1 and GluR2 positive neurons were seen in the three trigeminal divisions of the TG and they were independent of the soma sizes (Figs. 1(A and B) and 2). GluR2 positive neurons were more frequently encountered than GluR1 positive neurons; 638 and 113 per TG, respectively (five representative sections per TG). Both GluR1 and GluR2 subunits were stained predominantly in small (>400 μm^2; 46.8 and 31.25%, respectively) to medium size cells (400–1000 μm^2; 50.8 and 57.85%, respectively), but the percentage of large cells (<1000 μm^2) stained for GluR2 was higher than that of GluR1 (10.9 and 2.4%, respectively).

Masseter injections with 2% FB produced robust labeling of muscle afferents in TG within 5–7 days after the injection. The retrograde labeling of FB was limited to the mandibular division of TG and appeared to label small to medium size TG neurons. The mean area of 185 FB labeled cells from representative TG sections was 452 ± 17 μm^2, ranging from 115 to 1312 μm^2. FB labeled neurons could also be found in the trigeminal motor nucleus while there were no labeled cells in the facial motor nucleus, suggesting that the tracer did not leak out to overlying cutaneous tissue. Both GluR1 and GluR2 subunits were localized in FB positive muscle afferents. The percentage of GluR2 positive muscle afferents was significantly higher than that of GluR1 positive muscle afferents in TG (61% ± 11.5 and 20.25% ± 7.2, respectively; $t = 6.004$,