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

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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 seconds 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, Schwann 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

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(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 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 μ l) 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 μ m. 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 non-specific background staining. GluR1/2 positive cells were counted from representative sections obtained from four TG. Labeled TG neurons were classified as small (>400 μ m²), medium (400–1000 μ m²), and large cells (<1000 μ m²) (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 μ l; Tocris Cookson), or vehicle control (PBS) in the masseter 5 minutes prior to MO

(20% in 30 μ l) injection in the same muscle. Another group of rats (n=8) received the highest dose of NBQX (100 nmol) in the forelimb muscle 5 minutes prior to MO injection in the masseter to control for possible systemic effects of NBQX.

We have previously shown that algescic stimulation of the masseter muscle evokes ipsilateral hind-paw 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-sec intervals. The counts were plotted over time to determine the temporal pattern of response. The highest number of shakes occurring during the first 30-sec 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 $\pm$ 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 two hours 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 μ m) 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 hr), and avidin-biotin-peroxidase complex (Vector Laboratory; 1 hr). 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 3–5 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 (Fig.1A,B; Fig.2). GluR2 positive neurons were more

frequently encountered than GluR1 positive neurons; 638 and 113 per TG, respectively (5 representative sections per TG). Both GluR1 and GluR2 subunits were stained predominantly in small ($>400\ \mu\text{m}^2$; 46.8 and 31.25%, respectively) to medium size cells ($400\text{--}1000\ \mu\text{m}^2$; 50.8 and 57.85%, respectively), but the percentage of large cells ($<1000\ \mu\text{m}^2$) 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 $452\pm 17\ \mu\text{m}^2$, ranging from 115 to $1312\ \mu\text{m}^2$. 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 ($61\%\pm 11.5$ and $20.25\%\pm 7.2$, respectively; $t=6.004$, $p<0.001$; $n=4$ TG). Examples of double labeled masseter afferents are shown in Figure 1C–H.

3.2 Effects of peripheral NBQX on MO-induced nocifensive behavior

Injection of PBS or NBQX prior to MO did not produce noticeable hindpaw responses. The group of animals pretreated with PBS in the masseter followed by MO injection exhibited a pattern of behavioral responses similar to that described for injection of MO alone (Ro et al., 2003; Fig. 3A). NBQX pretreatments in the masseter significantly attenuated the MO-induced hindpaw responses. The MPC of NBQX pretreated rats were significantly lower than those of the vehicle treated rats with a significant difference between the vehicle and NBQX (50 and 100 nmol) pre-treated groups ($F=12.4$, $p<0.001$; Fig. 3B). There was also a significant difference of AUC between the vehicle and NBQX (50 and 100 nmol) pre-treated groups. However, the NBQX effect was less robust in statistical comparisons of AUCs ($F=3.2$, $p<0.05$; Fig. 3C). The significant NBQX effect seen at 50 nmol was not further enhanced with a two-fold higher dose at 100 nmol for either MPC or AUC. The pretreatment of the forelimb muscles with the highest dose of NBQX (100 nmol) did not have a significant effect on either the magnitude or duration of the ipsilateral hindpaw shaking responses.

3.3 Effects of peripheral NBQX on MO-induced Fos-LI in the Vc

In the rats that received PBS followed by MO, intense Fos-LI was consistently seen in ipsilateral laminae I and II of the caudal Vc (Fig. 4). There was little Fos-LI in deeper laminae, and Fos-LI in the contralateral Vc was minimal, a pattern similar to that following stimulation of the masseter with MO alone (Ro et al., 2003). Consistent with the behavioral data, NBQX pretreatment significantly reduced the average number of Fos positive neurons in the ipsilateral caudal Vc ($F=4.29$, $p<0.01$; Fig. 5A). The highest dose of NBQX (100 nmol) did not further enhance the significant effect on the average Fos counts produced with 50 nmol of NBQX. The highest dose of NBQX administered to the forelimb muscle did not significantly reduce the number of MO-induced Fos positive neurons, which confirms the behavioral data that the NBQX effect is primarily mediated by local AMPA/KA receptors in the masseter. Neuronal activation was minimal in the contralateral Vc of all groups (Fig. 5B).

4. DISCUSSION

4.1 AMPA receptor subunits are expressed in trigeminal primary afferents

Early studies that report the presence of glutamate receptors in both peripheral and central terminals of primary afferent neurons implicate their contribution to the regulation of sensory input from the earliest stage of information processing (Lee et al., 1999, 2002). AMPA

receptors are one type of ionotropic glutamate receptors that are involved in fast glutamatergic transmission in the CNS. Functional AMPA receptors are formed by heteromeric assemblies of four subunits, GluR1 through GluR4 (Hollmann and Heinemann, 1994). All four AMPA subunits have been identified in rat dorsal root ganglion (DRG) neurons (Chambille and Rampin, 2002; Lu et al., 2002), with heterogeneous subunit compositions: small DRG neurons express GluR1/GluR2,3 while large DRG neurons lack GluR1 expression (Sato et al., 1993). AMPA receptors in peripheral terminals of primary afferent neurons are associated with clusters of small vesicles, and AMPA receptors at this location have been suggested to function as auto-receptors as have been shown for NMDA receptors at central terminals of primary afferent neurons (Liu et al., 1994; Carlton et al., 1995). The ultra-structural observation that GluR1 is mainly present in unmyelinated cutaneous axons strongly suggests that modulation of nociceptive input by peripheral glutamate involves AMPA receptors (Carlton et al., 1995; Coggeshall and Carlton, 1998).

GluR1, 2, and 3, have also been demonstrated in sensory neurons of the trigeminal system (Quartu et al., 2002; Lee and Ro, 2007b). In human TG, GluR2/3 subunits are expressed in all size of neurons suggesting the involvement of peripheral AMPA receptors in processing low threshold input in addition to nociceptive input (Quartu et al., 2002). This suggestion is supported by the localization of GluR1-4 in the rat mesencephalic nucleus of the trigeminal nerve (Mineff et al., 1998), a nucleus composed of cell bodies of muscle spindle and periodontal mechanoreceptive afferents (Capra and Dessem, 1992). Our observation that GluR2 is more abundantly expressed than GluR1 is consistent with the rat DRG and human TG data that GluR2 is expressed in all size of neurons while large DRG neurons lack GluR1 expression (Sato et al., 1993; Quartu et al., 2002). A higher percentage of GluR2 positive masseter afferents may reflect the prevalence of GluR2 positive TG neurons in general. However, since TG muscle afferents are mainly groups III and IV fibers (Capra and Wax, 1989), it is likely that GluR1 as well as portions of GluR2 positive masseter afferents are nociceptive afferents. The presence of GluR1/2 on small diameter masseter primary afferents provides a morphological basis for a functional role of AMPA receptors in the well documented pain and hyperalgesic responses following glutamate stimulation of the craniofacial muscle (Cairns et al., 2001, 2002; Svensson et al., 2003).

4.2 Effects of peripheral NBQX on MO-induced nocifensive behavior

Mechanical allodynia and thermal hyperalgesia produced by intraarticular injections with combinations of excitatory amino acids are significantly blocked when either AP7 or CNQX is co-administered (Lawand et al., 1997). Attenuation of inflammation-induced tissue hypersensitivity by AP7 and CNQX after full development of knee joint inflammation suggests both NMDA and AMPA/KA receptors play a pivotal role in the initiation as well as maintenance of acute inflammatory pain. Similarly, local pre-treatment with either MK-801 or CNQX equally blocks formalin-induced nocifensive behaviors and carageenan-induced thermal hyperalgesia in the rat hindpaw (Jackson et al., 1995; Davidson et al., 1997). These findings provide support for a role of peripherally localized NMDA as well as non-NMDA glutamate receptors in pain and hyperalgesia.

When mechanical responses were assessed two days after the intraplantar injection of CFA, only MK801, but not CNQX, significantly and dose-dependently attenuated CFA-induced mechanical hyperalgesia, data suggesting that hyperalgesic responses resulting from a chronic type of inflammation involves peripheral activation of NMDA, but not AMPA/KA receptors (Leem et al., 2001). However, it has to be noted that the dose range of CNQX used in the study by Leem et al. (2001) is an order of magnitude lower than that used in the studies described above. In a neuropathic pain condition, another AMPA/KA receptor antagonist, NBQX, failed to prevent the development of mechanical hyperalgesia when administered locally prior to

spinal nerve ligation, and did not reverse the mechanical hyperalgesia when given 10 days after the nerve ligation (Jang et al., 2004). MK801 effectively blocked the development of mechanical hyperalgesia and transiently reversed the nerve injury-induced mechanical hyperalgesia under the same conditions. Taken together, these studies suggest that all subtypes of peripheral ionotropic glutamate receptors contribute to acute pain conditions and that relative involvement of these receptors may vary under chronic inflammatory and neuropathic pain conditions.

Involvement of peripherally localized NMDA receptors in acute muscle pain conditions has been documented in both animal and human experimental studies (Cairns et al., 2003, 2006; Ro, 2003; Ro et al., 2007). In a more chronic condition, pre-treatment of the masseter with AP5 also significantly attenuates CFA-induced masseter hyperalgesia that last up to three days (Ro et al., 2005). The involvement of AMPA/KA receptors in orofacial pain conditions was suggested when co-application of CNQX reduced glutamate- and AMPA-evoked responses without significantly affecting NMDA-evoked responses (Cairns et al., 1998). The present finding that local NBQX significantly and dose-dependently reduced MO-induced nocifensive behavior further corroborate the notion that peripheral AMPA/KA receptors are equally important as NMDA receptors in acute pain conditions. However, specific contribution of either peripheral AMPA or KA receptors cannot be assessed since NBQX blocks both AMPA and KA receptors.

An interesting difference between the effects of local NMDA and AMPA/KA receptor antagonists on MO-induced behavioral responses is that the blockade of NMDA receptors produced a statistically more pronounced effect on overall magnitude of responses assessed as AUC (Ro, 2003), whereas the blockade of AMPA/KA receptors resulted in a more pronounced inhibition of MPC, which is measured within the initial minute. Thus, it is possible that AMPA receptors on muscle nociceptors contribute to initial responses to peripherally released glutamate whereas NMDA receptors provide prolonged responses so long as the glutamate is available. The data suggest that blockade of any one type of ionotropic glutamate receptors in the periphery may not always be sufficient to inhibit nociceptor activity. For example, the local ketamine, which acts as an NMDA receptor antagonist, was effective in relieving glutamate-induced muscle pain in male subjects, but failed to do so in female subjects (Castrillon et al., 2007). Since the masseteric injection of glutamate evokes a greater pain in female subjects (Svensson et al., 2003), it is possible that peripheral AMPA receptors play a greater role in mediating acute pain in female subjects. Thus, it is important to consider the combined contributions of all different subtypes of peripheral glutamate receptor, including mGluR, in developing therapeutic alternatives that target local glutamate receptors in treating muscle pain conditions.

4.3 Effects of peripheral NBQX on MO-induced c-fos activation in the Vc

Blockade of peripheral NMDA receptors reduces responses of wide dynamic range (WDR) neurons in the spinal cord dorsal horn to both noxious and innocuous stimuli (Ushida et al., 1999), suggesting glutamate receptors at peripheral nerve terminals provide sufficient input to activate and sensitize central neurons (Wang et al., 1997; Ushida et al., 1999). Hyperexcitability of cat dorsal horn WDR neurons resulting from local injections of bee venom is prevented when AP5 or CNQX is pre-administered locally (Chen et al., 1999). In contrast, local pre-treatment with AP5, but not CNQX, prevents the occurrence of the increased firing of WDR neurons following bee venom injection in rats (You et al., 2002). This discrepancy could be due to species differences. Nevertheless, these data reinforce the notion that persistent activity arising from nociceptors lead to hyperexcitability and neuroplastic changes in the spinal dorsal horn neurons (Cook et al., 1987; Hylden et al., 1989), and suggest that peripheral NMDA and AMPA/KA receptors play a critical role in this process.

In our previous studies, we proposed that peripheral NMDA receptors contribute significantly to activation of central trigeminal neurons induced by algescic stimulation of craniofacial muscle based on the evidence that local MK-801 treatment significantly and dose-dependently attenuated hypertonic saline- and MO-induced Fos expression in the Vc, especially in the caudal Vc (Ro et al., 2004, 2007), an area that contains the highest density of NMDA receptors with the greatest input from small diameter muscle afferents (Shigenaga et al., 1988; Tallaksen-Greene et al., 1992). The present study provided new evidence that peripheral AMPA/KA receptors also contribute to activation of central trigeminal neurons. A major advantage in the design of the present study was the ability to correlate behavioral responses with neuronal responses to the same stimulus from the same set of animals, and consistent data from both behavioral and Fos experiments further strengthen our proposal that peripheral AMPA/KA receptors play a significant role in acute muscle pain conditions. Future studies on their role in chronic types of muscle pain conditions are required. These results clearly suggest that multiple peripherally located glutamate receptor subtypes are activated in parallel following muscle tissue injury, and assessing the relative contribution from each receptor subtype under various pathological conditions will prove beneficial for treatment of craniofacial muscle pain.

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Figure 1. AMPA receptor subunits, GluR1 and GluR2, are expressed in trigeminal ganglion neurons (A, B). The somata of masseter afferents labeled by retrograde transport of Fast Blue (FB; arrow in C and in F) expressed GluR1 (arrow in D,E) or GluR2 subunit (arrow in G,H). Scale bar = 25μm.

Figure 2.

Soma size distribution of GluR1 (A) and GluR2 (B) positive neurons in TG. The histograms illustrate the distribution of GluR1 and GluR2 positive neurons based on cell body area (μm^2). The histograms were constructed from area measurements of 450 GluR1 and 1,275 GluR2 positive TG neurons.

Figure 3.

Effects of masseteric NBQX pre-treatment on MO-induced nocifensive behavior. Line graphs in A show the time course of MO-induced nocifensive hindpaw responses from vehicle and NBQX pre-treated rats. Bar graphs show the quantification of hindpaw responses as MPC and AUC (B,C). Data are shown as mean $\pm$ SE. ** $p < 0.01$, and * $p < 0.05$.

Figure 4.

Examples of Fos-LI in the caudal Vc. Photomicrographs show MO-induced Fos-LI in animals pre-treated with (A) PBS, (B) NBQX (1 nmol), (C) NBQX (100 nmol) in the masseter, and (D) NBQX (100 nmol) in the forelimb muscle. These photomicrographs were taken from the Vc region approximately 3 mm caudal from the obex. M-medial, L-lateral, D-dorsal, V-ventral. Scale bar = 25 μ m.

Figure 5. Effects of masseteric NBQX pre-treatment on mean number of cells with Fos-LI in the Caudal Vc. Masseteric pre-treatment with NBQX (50, 100nmol) produced significantly less number of Fos-LI in the ipsilateral Vc. Neurons in the contralateral Vc were rarely activated in any of the treatment conditions. ** $p < 0.01$.