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Differential regulation of glutamate receptors in trigeminal ganglia following masseter inflammation

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Abstract

The present study examined whether *N-methyl-D-aspartate* receptor (NMDAR) and 5- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) subunits and group I metabotropic glutamate receptors (mGluRs) are constitutively expressed in trigeminal ganglia (TG) using Western blot analysis in male Sprague Dawley rats. We then investigated whether experimental induction of masseter inflammation influences glutamate receptor expressions by comparing the protein levels from naïve rats to those from complete Freund's adjuvant (CFA) inflamed rats. Our results showed that NMDAR1 (NR1), NMDAR2A (NR2A), NMDAR2B (NR2B) subunits, and AMPAR1 (GluR1) and AMPAR2 (GluR2) subunits, and a group I metabotropic glutamate receptor, mGluR5, are constitutively expressed in TG. Masseter inflammation significantly down-regulated NR1 subunit expression that persisted to 7 days post CFA inflammation. NR2A and NR2B expressions were not significantly changed. GluR1 receptor subunit expression slightly increased in TG 3 days after CFA-induced inflammation, but the change was not statistically significant. GluR2 protein level was not affected by CFA inflammation. The level of mGluR5 protein was significantly up-regulated in TG 3 days after CFA-induced masseter inflammation. There were no inflammation-induced changes in any of the proteins we analyzed in the contralateral, non-inflamed TG. These results suggested that muscle inflammation differentially modulates glutamate receptor subunits at the primary afferent level in male rats and that these inflammation-induced transcriptional changes may contribute to functionally different aspects of craniofacial muscle pain.

INTRODUCTION

Functional contribution of peripherally localized glutamate receptors in acute and chronic pain processing is amply documented (5). The presence of various ionotropic and metabotropic glutamate receptor (mGluR) proteins in small sensory neurons corroborates the notion that peripheral glutamate receptors play a significant role in nociceptive processing (3,7,16). Recent studies demonstrate that functional compositions of glutamate receptor subunits in primary sensory neurons vary depending on cell types. For example, it has been shown that all *N-methyl-D-aspartate* receptor (NMDAR) subunits, NMDAR1 (NR1), NMDAR2A (NR2A), NMDAR2B (NR2B), NMDAR2C (NR2C), and NMDAR2D (NR2D), are expressed in dorsal root ganglia (DRG), but NR2D forms functional NMDA receptors only in C-fibers whereas

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NR2B is present in both A- and C-fibers (17). Subunit compositions for 5- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) are also heterogeneous among DRG neurons; small DRG neurons express AMPAR1/AMPA2,3 (GluR1/GluR2, 3) while large DRG neurons lack GluR1 expression (21). Type I mGluRs (mGluR1, mGluR5) that have been implicated in pro-nociceptive role are expressed in cell body as well as peripheral terminals of DRG neurons (6,22).

Peripheral inflammation following injection of complete Freund's adjuvant (CFA) in the hind paw produces a significant increase in the proportion of NR1-labeled unmyelinated axons (9). Experimental colitis specifically up-regulates NR2B subunit expression without affecting other NR2 subunits (15). It has also been shown that mGluR1 expression in DRG is down-regulated following peripheral nerve injury (11). These data suggest that peripheral glutamate receptors undergo different transcriptional regulations following inflammation or injury, and these changes may contribute to altered functional properties of primary afferent neurons under various pathological conditions.

Despite the accumulating data on the role of peripheral glutamate receptors in orofacial muscle pain (13), there is little information on the presence of ionotropic glutamate receptor subunits and mGluRs in trigeminal primary afferent neurons, and how muscle inflammation modulates expression of glutamate receptor proteins in trigeminal ganglia (TG). The objectives of this project were to demonstrate the expression of NMDA and AMPA receptor subunits as well as mGluR proteins, and to investigate whether CFA-induced masseter inflammation produces changes in the level of their expression in TG.

MATERIALS AND METHODS

Experiments were carried out on male Sprague-Dawley rats weighing between 280 to 350g. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For induction of masseter inflammation, rats were injected with 50 μ l of CFA (Sigma; dissolved 1:1 in isotonic saline) to the mid-region of masseter muscle. The relative expression of protein of interest from naïve rats was compared to those from the rats treated with CFA in the masseter muscle, 1, 3, and 7 days following the CFA injection. All experimental and control groups consisted of 5 rats per group. TG from both sides were extracted and Western blot analysis was performed as the following. Total proteins in TG were dissolved in RIPA buffer containing protease inhibitor cocktail. The protein concentration in lysates was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Thirty microgram of protein for each sample were fractionated on 10% NuPAGE gel with running buffer containing SDS and transferred to a PVDF membrane (Bio-rad, Hercules, CA). After blocking 1 h in 5% milk PBS at room temperature, membranes were probed with primary antibodies for glutamate receptor subunits and β -Actin, used as an internal control protein, diluted in blocking solution. The following primary antibodies were used: anti-NR1 (1:500 Santa-cruz), anti-NR2A (1:500 Santa-cruz), anti-NR2B (1:500 Santa-cruz), GluR1 (1:500 Santa-cruz), GluR2 (1:500 Santa-cruz), and anti-mGluR5 (1:1000, Upstate). mGluR1 was not included in this study since we have previously reported that mGluR1 protein is not reliably detected in TG (14).

Membranes were incubated with primary antibodies overnight at 4 °C and washed three times with PBS. HRP-conjugated secondary antibodies were diluted to 1:5000 in PBS and incubated with membranes for 1 h. Bands were visualized using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Protein level for each receptor subunit was normalized to that of β -Actin in the same sample. Data from CFA-inflamed rats were normalized to that of naïve rats and expressed as mean percent changes $\pm$ standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA)

followed by Duncan's multiple posthoc comparisons, and differences were considered significant at $P < 0.05$.

RESULTS

Immunoblots from TG homogenates showed a constitutive expression of NR1, NR2A, and NR2B subunits (Fig 1A, B, C; lane 1). Masseter inflammation caused a gradual down-regulation of NR1 expression that reached a statistically significant decrease by Day 3 and persisted to Day 7 (Fig 1A; $F=8.053$, $p=0.002$). There was a trend of similar down regulation in the contralateral TG, but the change was not statistically significant ($F=1.853$, $p=.178$). There was no significant change in protein levels for either NR2A or NR2B subunits (Fig 1B,C; $F=0.735$, $p=0.547$; $F=0.140$, $p=0.935$, respectively). Similarly, inflammation did not significantly alter the expression of either of the NR2 receptor subunits in TG contralateral to the side of inflammation ($F=0.239$, $p=0.995$; $F=0.631$, $p=0.649$, respectively).

Our results also showed the presence of GluR1 and GluR2 proteins in TG of naïve rats (Fig 2A, B; lane 1). However, there were no inflammation related changes in the expression level of either GluR1 or GluR2 protein in TG of both inflamed (Fig 2 A, B; $F=1.144$, $p=0.361$; $F=0.635$, $p=0.063$, respectively) and non-inflamed sides (data not shown). Consistent with our previous study, mGluR5 protein was reliably detected in TG of naïve rats (Fig 3A; lane 1). Masseter inflammation resulted in a statistically significant up-regulation of mGluR5 protein in a time dependent manner (Fig 3B, *left*; $F=3.359$, $p=0.045$). The level of mGluR5 protein in TG of inflamed side was significantly up-regulated 3 days following CFA treatment compare to that of naïve rats. There was no change in the level for mGluR5 protein in TG in the non-inflamed side under the same condition (Fig 3B, *right*; $F=1.981$, $p=0.144$). However, the lack of inflammation-induced changes in some glutamate subunit expressions needs to be interpreted with caution due to the small sample size in each group.

DISCUSSION

In the present study, we demonstrated that NMDA receptor subunits, NR1, NR2A, NR2B, and AMPA subunits GluR1 and GluR2 are constitutively expressed in the rat TG, and confirmed our previous finding of mGluR5 expression (14). Although all known NMDA and AMPA receptor subunits have not been examined in this study, these results provide evidence for functional compositions of ionotropic glutamate receptors in sensory ganglia that innervate orofacial tissue, and provide a proteomic basis for the well documented role of peripheral NMDA receptors in orofacial pain processing (4,8,19,20).

In addition, we demonstrated here that inflammation differentially modulates the expression of ionotropic glutamate receptor subunits and mGluRs. A significant down-regulation of NR1 subunit expression shown in our model of inflammation can alter the level of all NMDA receptors since NR1 subunit is required for functional NMDA receptor composition (18). Functional implications of inflammation-induced changes in NMDA receptor subunit expressions in TG can only be speculated at present. However, a similar prolonged reduction of NR1 subunit expression in DRG following hindpaw inflammation has been reported and suggested to contribute to a hypo-sensitization of primary afferent neurons (23). In another study, CFA-induced inflammation significantly increases the percentage of NR1-labeled unmyelinated axons in the digital nerve from 2 to 7 days following hindpaw inflammation before returning to the baseline level in 14 days (9). A possible explanation for this discrepancy is that inflammation facilitates axonal transport of NR1 subunits from soma to nerve terminals, thereby producing hypersensitivity, rather than hyposensitivity, of primary afferent nociceptors.

Our data indicate that NR1 and NR2 subunit expression in TG are under different transcriptional regulations. It was interesting to observe such a profound and prolonged reduction of NR1 subunit expression following masseter inflammation since masseter afferents represent only a small portion of TG neurons (1). It is possible that muscle inflammation can induce widespread and generalized reduction of NR1 subunits in TG neurons while selectively affecting NR2 subunit expressions only in masseter afferents. In DRG, an activity dependent release of diffusible substances from neuronal somata can partly influence neighboring cells (2). This mechanism has been proposed as a way in which most DRG neurons participate in ongoing mutual interactions. If this is the case, substances released from TG cell bodies could have had more global influence on NR1 subunit expression, but not on other NMDA receptor subunits. Thus subtle changes in NR2A and NR2B subunit expressions in masseter afferents could have been masked in the whole TG preparation. It has to be noted that Western blot experiments cannot provide information on localization of glutamate receptor expressions on specific population of TG afferents. Immunohistochemical studies are needed to address whether NMDA receptor subunits are expressed on TG afferents that innervate masseter muscle and how masseteric inflammation modulates their expression.

Data on inflammation-induced changes in AMPA receptor subunits in sensory neurons is limited. In this study, unlike NMDA receptor subunits, neither GluR1 nor GluR2 expression was significantly modulated in TG following masseter inflammation. Hindpaw inflammation significantly increases GluR1 and GluR2 immunoreactivity in the spinal cord and GluR1 immunoreactivity in rostral ventromedial medulla (10,24), indicating that inflammation modulates AMPA receptor expressions at different levels of nociceptive circuitry. Although not significant a similar tendency of increase in GluR1 subunit expression in TG following masseter inflammation suggests a potential contribution from peripherally located AMPA receptors in nociceptive processing under inflammatory conditions.

In spinal system, both mGluR1 and 5 are synthesized in DRG and transported to central as well as to peripheral terminals (3,22). We have previously shown that mGluR5, but not mGluR1, protein is reliably detected in TG and the masseter nerve (14). Here we provide additional information showing that masseter inflammation significantly up-regulates mGluR5 protein expression in TG. The mGluR5 up-regulation peaked at 3 days after inflammation and then returned to baseline in 7 days. Spinal nerve injury also produces up-regulation of mGluR5 protein in lumbar DRG neurons when examined two weeks after the injury (12). Since mGluR5 up-regulation we observed in TG occurred at a time point when NR1 subunit expression was significantly down-regulated, it would be interesting to study if there are concurrent changes in the level of both NR1 and mGluR5 proteins in peripheral terminals of the masseter nerve in order to understand functional contribution of these peripheral glutamate receptors in muscle pain processing.

In summary, we have shown that NMDA and AMPA receptor subunits and mGluR5 are constitutively expressed in TG and that muscle inflammation differentially modulates the expression of glutamate receptors in male rats. These changes in peripheral glutamate receptor expressions may contribute to functionally different aspects of craniofacial muscle pain processing under inflammatory conditions.

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Fig 1.

Expression of NMDA receptor subunits (NR1, NR2A and NR2B) in TG of naïve and CFA-inflamed rats (A, B, C). The bar graph shows relative levels of the NMDA receptor subunit proteins from the TG of inflamed and the contralateral sides. The protein level of NR1 subunit in the TG of inflamed side showed significant changes in CFA-inflamed rats 3 and 7 days following CFA treatment compare to that of naïve rats. * denotes significance at $P < 0.05$.

Fig 2.

Expression of AMPA receptor subunits (GluR1 and GluR2) in TG of naïve and CFA-inflamed rats (A, B). The bar graph shows relative levels of the AMPA receptor subunit proteins in the TG inflamed side (D,E). The data from the TG of side contralateral to the inflammation is not shown.

Fig 3.

Expression of mGluR5 protein in TG of naïve and CFA-inflamed rats (A). The bar graph shows the relative level of mGluR5 protein in TG of naïve and CFA-inflamed rats (B). The protein level for mGluR5 was significantly up-regulated in the TG of CFA-inflamed rats 3 days after CFA treatment. There was no significant change in the protein level of mGluR5 in the TG contralateral to the side of inflammation. * denotes significance at $P < 0.05$.