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Activation of TRPV1 and TRPA1 leads to muscle nociception and mechanical hyperalgesia

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Abstract

The involvement of TRPV1 and TRPA1 in mediating craniofacial muscle nociception and mechanical hyperalgesia was investigated in male Sprague Dawley rats. First, we confirmed the expression of TRPV1 in masseter afferents in rat trigeminal ganglia (TG), and provided new data that TRPA1 is also expressed in primary afferents innervating masticatory muscles in double-labeling immunohistochemistry experiments. We then examined whether activation of each TRP channel in the masseter muscle evokes acute nocifensive responses and leads to the development of masseter hypersensitivity to mechanical stimulation using the behavioral models that have been specifically designed and validated for the craniofacial system. Intramuscular injections with specific agonists for TRPV1 and TRPA1, capsaicin and mustard oil (MO), respectively, produced immediate nocifensive hindpaw responses followed by prolonged mechanical hyperalgesia in a concentration-dependent manner. Pretreatment of the muscle with a TRPV1 antagonist, capsazepine, effectively attenuated the capsaicin-induced muscle nociception and mechanical hyperalgesia. Similarly, pretreatment of the muscle with a selective TRPA1 antagonist, AP18, significantly blocked the MO-induced muscle nociception and mechanical hyperalgesia. We confirmed these data with another set of selective antagonist for TRPV1 and TRPA1, AMG9810 and HC030031, respectively. Collectively, these results provide compelling evidence that TRPV1 and TRPA1 can functionally contribute to muscle nociception and hyperalgesia, and suggest that TRP channels expressed in muscle afferents can engage in the development of pathologic muscle pain conditions.

Keywords

Trigeminal ganglia; muscle nociceptors; craniofacial; rat; behavior

1. Introduction

Chronic muscle pain conditions, such as temporomandibular disorders (TMD), are characterized by localized myalgia and tenderness of the muscle upon manual palpation [23]. These conditions are also associated with reduced maximum force output and limited range of movements [36,41], which could result from sensitization of muscle nociceptors [39]. Thus,

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There are no conflicts of interest.

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prominent features associated with muscle pain condition are changes in mechanical sensitivity of muscle tissue. However, the pathophysiological mechanisms leading to the development of mechanical hyperalgesia are still poorly understood.

Recent studies indicate that several members of the transient receptor potential (TRP) family, namely TRPV1 and TRPA1 function as either sensory transducers for noxious mechanical stimuli or play an essential role in the development of mechanical hypersensitivity under various pain conditions [35]. Both TRP channels are expressed in distinct subgroups of primary afferent neurons: TRPV1 is predominantly expressed in small diameter dorsal root ganglia (DRG) and TG neurons [12,27] and TRPA1 in a subset of TRPV1 containing neurons [9,29, 51]. While data on cellular and molecular mechanisms of each of these channels have been accumulating [16,17,37,46,57] our knowledge of the functional contributions of these channels in muscle pain conditions is limited. Given the clinical relevance of the mechanical nature of muscular hyperalgesia it becomes pertinent to evaluate specific contributions of these TRP channels and the cellular mechanisms underlying the development and maintenance of such conditions.

TRPV1 is expressed in a small subpopulation of muscle afferents in DRG, and direct injection with a TRPV1 agonist, capsaicin, activates muscle nociceptors while pharmacological blockade of TRPV1 reduces exercise-induced muscle hyperalgesia in the rat [19,24]. Intramuscular capsaicin in human subjects activates muscle nociceptors and evokes an intense deep pain sensation as well as mechanical hyperalgesia [5,6,38]. Capsaicin injection into the masseter muscle evokes mechanical hypersensitivity in rats [50] suggesting functional TRPV1 channels are expressed in masseteric afferents. Similarly, masseteric injections with a potent TRPA1 agonist, mustard oil (MO), evokes concentration-dependent nocifensive responses and inflammation in rats [33,49], also implicating TRPA1 in pain arising from craniofacial deep tissue. While TRPV1 is shown to be expressed in a subpopulation of masseteric afferents [54] there are no data on whether TRPA1 is similarly expressed in afferents innervating limb or craniofacial muscles.

In this study, we examined (1) the expression level of both TRPV1 and TRPA1 in muscle afferents in TG, (2) the effects of direct intramuscular administration of an agonist for each TRP channel on acute nocifensive behavior and mechanical hyperalgesia, and (3) investigated whether agonist-induced behavioral responses are specifically mediated by the TRP channels.

2. Materials and Methods

2.1 Experimental 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 TRP channels in masseter afferents

Fast Blue (FB) (2%; 10µl) was injected in the masseter to retrogradely label muscle afferents in TG of three rats. FB was injected into multiple sites in the masseter muscle using aseptic techniques. To avoid any leakage of the tracer the injection needle was left in place for 1-2 min before it was slowly retracted. The injection site was then covered with petroleum jelly. 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). The right TG from each rat was extracted and post-fixed for 90 min, placed in 30% sucrose

solution at 4°C overnight and sectioned coronally at 12 µm. Every fourth section was collected and mounted on gelatin-coated slides for double-labeling immunohistochemistry. The sections were incubated overnight with primary antisera for TRPV1 (1:1000; rat polyclonal; Neuromics), and TRPA1 (1:1500; rat polyclonal, Neuromics). 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 for each TRP channel was omitted from the processing of selected sections to control for non-specific background staining.

TRP and FB positive cells were counted from 12 representative sections per ganglion from three TG. The somatotopic distribution of FB positive cells within the TG were assessed to assure that FB did not spread to other divisions of the TG. Trigeminal and facial motor nuclei were also evaluated as positive and negative control for FB labeling, respectively. Only the labeled neurons that showed clear nucleolus were included in the counting. Labeled TG neurons were classified as small ($>400\text{ }\mu\text{m}^2$), medium ($400\text{--}1000\text{ }\mu\text{m}^2$), and large cells ($<1000\text{ }\mu\text{m}^2$) [27]. The soma sizes were measured from labeled neurons that showed clear nucleolus. Percentages of masseter afferents double labeled with TRPV1 and TRPA1 were calculated and presented as mean $\pm$ standard error of the mean (SE).

2.3 Behavioral Studies and Data Analysis

We have previously shown that algesic stimulation of the masseter muscle evokes immediate ipsilateral hindpaw shaking responses in lightly anesthetized rats and validated that the behavioral responses reflect acute craniofacial muscle nociception [49]. Using this behavioral paradigm we quantified the TRP channel agonist-induced hindpaw shaking responses as a measure of acute nocifensive responses. Initially, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and a tail vein cannulated. Once the animals reached the 'light' anesthetic level, by showing withdrawal responses to every tail pinch with a clip calibrated to produce 600 gm of force, the venous line was connected to an infusion pump for continuous infusion of pentobarbital. The rate of infusion was adjusted to maintain a relatively light level of anesthesia throughout the duration of experiment (3–5 mg/h).

The agonist-induced hindpaw responses were quantified by counting the number of shakes in 30 sec intervals. The hindpaw responses were captured by a digital video camcorder for off-line verification of the counts. The counts were plotted over time to determine the temporal pattern of response. Total counts of hindpaw shakes over the observation period (usually lasting 2–3 minutes following the injection) were assessed as a measure of overall magnitude of response.

We have also demonstrated that noxious mechanical stimulation over the masseter muscle reliably evokes hindpaw responses, and that masseteric injections of widely used sensitizing agents significantly increase mechanical sensitivity of the muscle in a time dependent manner without affecting other muscles and overlying skin [32,48]. This behavioral paradigm allows us to deliver calibrated and controlled mechanical stimulations in the masseter muscle over an extended period of time (i.e., 1–2 hr). Here we have combined these behavioral models to assess whether activation of TRP channels invokes time-dependent changes in the mechanical sensitivity of the muscle tissue, in addition to assessing immediate nocifensive responses that occur within first few minutes, in the same animal.

A baseline mechanical threshold for evoking the hindpaw responses was determined 15 min prior to drug injection using the electronic von Frey (VF) anesthesiometer (IITC Life Science, Inc, Woodland Hills, CA). A rigid tip attached to the VF meter was applied to the masseter muscle until the animals responded with hindpaw shaking. We rested the animal's head flat against the surface of the table when pressing the anesthesiometer on the masseter in order to provide a stable set-up. The threshold was defined as the lowest force necessary to evoke the

hindpaw response. Changes in masseter sensitivity were then assessed at 15, 30 45, 60 and 90 min following drug treatments. Two readings were taken at 2 min intervals for each time point and the measures were averaged. The baseline mechanical threshold that evokes the hindpaw responses ranged from 500-650g in the lightly anesthetized rats. We calculated percent changes in VF thresholds following drug treatment with respect to the baseline threshold and plotted against time. In order to assess the overall magnitude of drug-induced changes in masseter sensitivity over time, area under the curve (AUC) was calculated for the normalized data for each rat. All behavioral observations were made by one experimenter blinded to experimental conditions to maintain the consistency of counting and assessing mechanical sensitivity.

2.4 Experimental and Control Groups

To test whether activation of each TRP channel evokes muscle nociception and mechanical hypersensitivity a specific agonist for TRPV1, capsaicin (0.1%, 0.01% and 0.001%), and a specific agonist for TRPA1, MO (10%, 5%, and 1%), and the vehicle for each agonist were administered directly into the masseter muscle. To determine that capsaicin or MO-induced behavioral responses are receptor-mediated the masseter muscle was pretreated with capsazepine (200 nmol), a specific antagonist for TRPV1, or AP18 (1 μ mol), a selective antagonist for TRPA1, 5 minutes prior to injections with the highest concentration of each agonist in the same muscle. The same dose of each antagonist was administered in the muscle contralateral to the capsaicin- or MO-treatment in additional groups of rats to confirm that the antagonists' effects are by mediated by blocking local TRP channels and not by systemic effects. In additional rats, each antagonist was administered without the intramuscular agonist injection to rule out the possibility that the antagonist alone can produce analgesic responses. Finally, in order to confirm that the capsaicin and MO-induced responses are specifically mediated by TRPV1 and TRPA1, another set of selective antagonists for TRPV1 and TRPA1, AMG9810 (100nmol and 1 μ mol) and HC030031 (1 μ mol and 10 μ mol), respectively, was administered in the masseter prior to the agonist treatment. Experimental and control groups were randomized and all groups consisted of 6 rats per group.

2.5 Drug Preparation and administration

Capsaicin (Sigma; 100 μ l) was dissolved in ethanol (20%), Tween 80 (7%) and PBS (70%) and MO (Fluka; 20 μ l) was diluted in mineral oil. Capsazepine (Sigma; 10 μ l) was prepared in DMSO (4%), Tween 20 (1%) and PBS (95%). AP18 (Biomol; 10 μ l), AMG9810 (Tocris; 10 μ l), and HC030031 (Biomol; 20 μ l) were dissolved in DMSO (100%).

In order to make sure that the drugs and vehicles are administered in the same target region of the muscle the injection site was determined by palpating the masseter muscle between the zygomatic bone and the angle of the mandible. Injections were made with a 27-gauge needle. Upon contacting the mandible the needle was slowly withdrawn into the mid-region of the masseter and injections were made for 5-10 seconds.

2.6 Statistical Analysis

For the behavioral data analysis, two-way ANOVA with repeated measures was used. In addition, one-way ANOVA was used to evaluate the overall magnitude of responses in total number of hindpaw shakes and AUC values for experimental and control groups. All multiple group comparisons were followed by a *post hoc* test (Dunnett's). The significance of all statistical analyses was set at $p < 0.05$.

3. Results

3.1 Both TRPV1 and TRPA1 channels are expressed in masseter afferents in TG

We have recently confirmed the expression of mRNAs for TRPV1 and TRPA1, as well as their protein products in the rat TG [34]. In this report, we provide immuno-histochemical evidence for expression of these TRP channels in TG neurons that innervate the masseter muscle (Fig 1). TRP positive neurons were seen in all three divisions of the TG. Both TRPV1 and TRPA1 expression was primarily observed in small to medium size cells. Mean soma areas for TRPV1 and TRPA1 positive TG neurons were $319 \pm 3.95 \mu\text{m}^2$ and $488 \pm 9.35 \mu\text{m}^2$, respectively. Size distribution for TG neurons positive for each TRP channel is shown in Figure 2.

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. 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 TRP channels were localized in FB positive muscle afferents. The percentages of TRPV1 and TRPA1 positive muscle afferents in TG were $23.7\% \pm 2.6$ and $10.8\% \pm 4$, respectively. Examples of double labeled masseter afferents are shown in Figure 1C and F.

3.2 Effects of TRPV1 activation on overt nociception and masseter hypersensitivity

Direct application of capsaicin in the masseter muscle resulted in immediate and intense hindpaw shaking responses that peaked within the first 30 seconds and subsided within 2 minutes (Fig 3A). The vehicle injection produced weak hindpaw responses that subsided within the first 30 second block. Two-way ANOVA yielded significant main effect for treatment ($F=6.1$; $p=0.006$) as well as time ($F=181.5$; $p<0.001$). The post-hoc analysis showed a significant effect of the capsaicin on the hindpaw responses at 30 sec and 60sec time points. Total hindpaw responses following capsaicin treatments increased in a concentration-dependent manner resulting in significantly higher numbers of total hindpaw responses in capsaicin treated rats (Fig 3B; $F=6.34$; $p=0.003$).

In addition to the immediate nocifensive hindpaw responses, capsaicin also induced time-dependent changes in noxious mechanical threshold of the masseter (Fig 3C). There were significant main effects for treatment ($F=16.95$; $p<0.001$) and time ($F=65.9$; $p<0.001$) on hindpaw responses. Significant capsaicin treatment effect was observed at 15, 30 and 45 min following the injection. Capsaicin (0.1%) treatment resulted in approximately 40% reduction of the threshold within the first 15 minutes that gradually returned to the baseline in 90 minutes. A similar reduction in mechanical threshold at the 15 minute time point following a lower concentration of capsaicin (0.01%) more quickly returned to the baseline level. The overall magnitude of masseter hypersensitivity measured as AUC showed a statistically significant effect (Fig 3D; $p<0.001$). Capsaicin treatments (0.1 and 0.01%) evoked significantly greater AUC compared to that of the vehicle treatment.

Pretreatment with capsazepine significantly attenuated capsaicin-induced total number of hindpaw nocifensive responses (Fig 3E; $F=9.726$, $p<0.001$). When the same dose of capsazepine was administered in the contralateral muscle the total hindpaw responses was not significantly different from that of the vehicle pre-treated capsaicin group suggesting that the capsazepine effect is mediated by blockade of local TRPV1 (Fig 3F). Similarly, capsaicin-induced masseter hypersensitivity was effectively blocked when capsazepine was pre-administered in the same muscle, but not in the contralateral muscle (Fig 3H; $F=10.9$, $p<0.001$). When administered alone capsazepine produced hindpaw responses that were similar to vehicle

injection and evoked mild hypersensitivity suggesting that capsazepine alone does not produce any analgesic effects.

In order to confirm that the capsaicin effect in the masseter is mediated via the TRPV1 receptors another selective TRPV1 antagonist, AMG9810, was pre-administered in the masseter prior to capsaicin injection (Fig. 5A-D). There were significant main effects for drug treatment ($F=6.1$; $p<0.05$) and time ($F=108.5$; $p<0.001$) on immediate hindpaw responses. Pretreatment of the masseter with 1 μmol of AMG9810 attenuated the capsaicin-induced total hindpaw responses, but the effect was not statistically significant (Fig 5B). There were also significant main effect for drug treatment ($F=18$; $p<0.001$) and time ($F=94.4$; $p<0.001$) on capsaicin-induced mechanical hyperalgesia. The capsaicin-induced mechanical hyperalgesia was partially, but significantly attenuated when the muscle was pre-treated with AMG9810 (1 μmol) (Fig 5C). AMG9810 (1 μmol) effectively reduced the overall magnitude of capsaicin-induced mechanical hyperalgesia as shown by significant reduction of the AUC value (Fig 5D; $F=8.9$; $p=0.003$).

3.3 Effects of TRPA1 activation on overt nociception and masseter hypersensitivity

Masseteric administration of MO produced concentration-dependent hindpaw responses in the rat. Mineral oil as vehicle control did not result in any noticeable hindpaw responses while MO reliably elicited the responses in the first minute following the injection (Fig 4A). There were significant main effects for treatment ($F=46.5$; $p<0.001$) and time ($F=50.7$; $p<0.001$) on hindpaw responses. Significant MO treatment effect was observed at 30 and 60 sec time blocks. Total hindpaw responses following MO increased in a concentration-dependent manner with 5% and 10% MO resulting in a significantly greater number of responses than that following the vehicle treatment (Fig 4B; $F=34.7$, $p<0.001$).

Intramuscular MO also induced a robust time-dependent increase in masseter sensitivity as revealed by significant main effects for treatment ($F=13.3$; $p<0.001$) and time ($F=72.2$; $p<0.001$) (Fig 4C). Significant MO treatment effect was observed at 15, 30 and 45 min following the injection. The magnitude and time course of changes in mechanical threshold following MO (10%) treatment were similar to that induced by capsaicin (0.1%) treatment. While the extent of reduction in mechanical threshold was lower with 5% than 10% MO treatment, responses to both concentrations of MO persisted for a prolonged period of time resulting in robust significant differences in AUC values when compared to that of the vehicle treated group (Fig 4D; $F=17.2$, $p<0.001$).

Pretreatment with AP18 produced a moderate, but significant, reduction of MO-induced hindpaw nocifensive responses (Fig 4E; $F=8.9$, $p<0.001$). The same dose of AP18 administered in the contralateral muscle did not result in significant attenuation of MO-induced hindpaw responses suggesting that the AP18 effect is mediated by blockade of local TRPA1 (Fig 4F). MO-induced masseter hypersensitivity was profoundly blocked when AP18 was pre-administered in the same muscle, but not in the contralateral muscle (Fig 4H; $F=32.2$, $p<0.001$). When administered alone AP18 produced minimal hindpaw responses and evoked mild hypersensitivity that returned to baseline in 30 minutes suggesting that AP18 alone did not produce any analgesic effects.

In order to confirm that the MO effect in the masseter is mediated via the TRPA1 receptors another selective TRPA1 antagonist, HC030031, was pre-administered in the masseter prior to capsaicin injection (Fig. 5E-H). There were significant main effects for drug treatment ($F=13.9$; $p=0.001$) and time ($F=83.1$; $p<0.001$) on immediate hindpaw responses. Pretreatment of the masseter with 10 μmol of HC030031 significantly attenuated the MO-induced total hindpaw responses (Fig 5F). There were also significant main effect for drug treatment ($F=33.2$; $p<0.001$) and time ($F=97.3$; $p<0.001$) on MO-induced mechanical hyperalgesia. The

MO-induced mechanical hyperalgesia was significantly blocked when the muscle was pre-treated with HC030031 (10 μ mol) (Fig 5G). HC030031 (10 μ mol) effectively reduced the overall magnitude of capsaicin-induced mechanical hyperalgesia as shown by significant reduction of the AUC value (Fig 5H; $F=41.9$; $p<0.001$).

4. Discussion

4.1 Expression of TRPV1 and TRPA1 in muscle afferents

Several studies have shown TRPV1 expression in small to medium size DRG neurons in the rat, but with varying proportions: over 45% express TRPV1 mRNA and anywhere between 30 to 54% stain for TRPV1 protein [1,8,11,22,29,40]. TRPV1 protein is expressed in 20-25% of rat TG, primarily in small to medium size neurons [27,54]. Available data on TRPV1 expression on muscle afferents also vary. TRPV1 is reported to be expressed in 5-25% of gastrocnemius muscle afferents in DRG [19,24], and 37.5% of masseter afferents in TG [54]. In this study, we confirmed that TRPV1 is expressed primarily in small to medium size cells in TG and provided additional information that approximately 24% of masseter afferents are TRPV1 positive. The difference between this study and the study by Takeda et al could be due to methodological differences such as the type of dye used and duration of retrograde transport from the muscle.

The expression level of TRPA1 in rat sensory neurons seems to be generally higher compared to that in mouse sensory neurons: TRPA1 protein is expressed in 20-30% and TRPA1 mRNA in 30-40% of DRG neurons in the rat whereas only 3.6% of mouse DRG neurons express TRPA1 transcript [8,17,29,42,51]. *In situ* hybridization analyses detected TRPA1 expression in 20-37% of rat TG neurons [28,29]. These studies indicate a significant species difference, but little difference between DRG and TG neurons, in TRPA1 expression. TRPA1 has been shown to be expressed in sensory neurons innervating smooth muscles and enteric nervous system [21,43], but there is no available data on its presence in sensory afferents innervating skeletal muscles. Thus, our data provide the first evidence for TRPA1 expression in masseter muscle afferents and its relative expression compared to those muscle afferents expressing TRPV1. In this study, we have not systematically examined whether TRPA1 positive neurons are a subset of TRPV1 positive muscle afferents. Given the fact that muscle afferents in TG are primarily small to medium size [2], and a similar somata size distribution between TRPV1 and TRPA1 positive TG neurons it is likely that TRPA1 and TRPV1 are co-expressed in masseter afferents.

4.2 TRPV1 and masseter pain and mechanical hypersensitivity

Mice lacking functional TRPV1 display normal physiological and behavioral responses to noxious mechanical stimuli; chemical and thermal hyperalgesia are markedly reduced while mechanical hyperalgesia develops under inflammatory conditions [10,13,14,16,18]. Mechanical hyperalgesia is attenuated in TRPV1 knock-out mice sixteen days after intraplantar injection of CFA in the hind paw and tail suggesting TRPV1 can participate in mechanical hyperalgesia associated with the chronic phase of CFA-induced arthritis [53].

Pharmacological or transcriptional modulation of TRPV1 in the rat provide more compelling support for the involvement of TRPV1 in the development or maintenance of mechanical hyperalgesia under various pain conditions. Application of the TRPV1 antagonist, BCTC, or antisense oligonucleotide against TRPV1 similarly reduces mechanical hypersensitivity in rats with spinal nerve ligation [15]. TRPV1 antagonists also effectively reduce capsaicin- or CFA-induced mechanical hyperalgesia [20,25,45]. Pretreatment with capsazepine prevented the development of capsaicin-induced mechanical hyperalgesia, with a similar potency in rat, mice and guinea pig [56]. Interestingly, the capsazepine pretreatment did not affect CFA-induced

mechanical hyperalgesia of the rat or mouse while producing a profound reversal in the guinea pig. Collectively, these studies clearly implicate TRPV1 in the development of mechanical hyperalgesia in animal models of inflammatory and neuropathic pain, and demonstrate species differences as a potential factor influencing the functional contribution of TRPV1.

Along with the data from the present study, others have shown that direct intramuscular injection of capsaicin significantly lowers noxious mechanical thresholds both in humans and rats [4,50], which could result from sensitization of polymodal acid/capsaicin sensitive TRPV1 receptors on group IV muscle afferents [24]. A recent study has demonstrated that blockade of TRPV1 effectively attenuates mechanical hyperalgesia developed following eccentric muscle contraction, but not the one after carageenan injection in the muscle [19]. Since the underlying mechanisms for the development of pain and hyperalgesia in the two types of muscle pain conditions are not clearly understood, and since only one time point after carageenan injection was tested for TRPV1 blockade, it is premature to conclude that TRPV1 is not involved in carageenan-induced mechanical hyperalgesia in the muscle tissue.

Comparable concentrations of capsaicin (0.01-0.1%) injected in human masseter produce considerably longer pain responses and mechanical hyperalgesia than the duration of capsaicin responses reported in this study [6]. There are, however, several important factors that could contribute to these differences. In addition to a species difference, the state of the subjects can be another major factor since the rats in this study were tested in 'lightly anesthetized' condition. A higher concentration of capsaicin (1%) injected in the rat masseter under halothane anesthesia evokes primary afferent discharge only for about 30 seconds [31]. Additional parameters such as injection volume and the size of the muscle could also be contributing factors. These potential factors do not warrant the direct comparisons between the rat and human data.

In the masseter muscle, it is well established that peripherally released excitatory amino acids (EAAs) contribute to overt nociception as well as to the development of mechanical hypersensitivity [47]. Despite the overwhelming amount of data implicating the pro-nociceptive and/or pro-inflammatory role of peripheral EAA receptors, mechanisms by which activation of these receptors lead to mechanical hypersensitivity remain unclear. Recent studies implicate functional coupling between peripherally localized glutamate receptors and TRPV1 as a potential mechanism underlying masseter pain and hypersensitivity [4,48]. In DRG neurons, activation of metabotropic glutamate receptor 5 (mGluR5) leads to increased thermal sensitivity by enhancing TRPV1 function via a cAMP-dependent intracellular signaling pathway [26]. Further studies on functional linkage between type I mGluR as well as NMDA receptors with TRPV1 as underlying factors for mechanical hypersensitivity in craniofacial muscle afferents are currently under investigation.

4.3 TRPA1 in masseter pain and mechanical hypersensitivity

The role of TRPA1 as a mechanical sensor in sensory neurons is controversial [52]. Mice lacking TRPA1 do not exhibit an unequivocal deficit in noxious mechanosensation [9,30]. However, TRPA1-deficient mice exhibit pronounced deficits in bradykinin-evoked nociceptor excitation and pain hypersensitivity [7,9], suggesting TRPA1 function as an important downstream transduction machinery through which inflammatory mediators produce pain and hyperalgesia. Recent studies provide evidence that bradykinin as well as other G-protein coupled receptors such as protease activated receptors enhance inflammatory pain responses by sensitizing TRPA1 via phospholipase C and PKA mediated intracellular signaling pathways [17,57]. A specific TRPA1 inhibitor, AP18, reverses CFA-induced mechanical hyperalgesia in normal but not in TRPA1 deficient mice suggesting that TRPA1 is required in the development of mechanical hyperalgesia under inflammatory settings [44].

We and others have shown that masseteric injections with MO in the rat evoke reliable nocifensive responses in a concentration-dependent manner [33,49]. In this study, we extended these observations by showing that masseteric MO also induces prolonged mechanical hypersensitivity in muscle tissue, effects that are specifically mediated via TRPA1 in muscle afferents. Considering a recent surge on data suggesting TRPA1 as a sensory receptor for multiple chemical mediators of inflammation, injury or oxidative stress [3,8,55], it is likely that TRPA1, along with TRPV1, contributes to pathological muscle pain responses under injury or inflammatory conditions.

In summary, our data showed that intramuscular capsaicin and MO induce overt nociception and mechanical hyperalgesia via TRPV1 and TRPA1 channels, respectively. The fact that capsaicin and MO-induced responses can be significantly blocked by two different sets of selective antagonists strongly suggests the functional involvement of both TRP channels in craniofacial muscle pain processing. The data in the present study also suggest that both TRPV1 and TRPA1 channels may be recruited and sensitized via distinct intracellular pathways by various inflammatory mediators, which could be manifested as overall muscle pain mechanical hyperalgesia in muscle tissue. Understanding mechanisms by which various pro-nociceptive receptors or channels engage TRP channels in producing mechanical hypersensitivity in muscle tissue is, therefore, of great clinical significance.

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Figure 1. TRPV1 and TRPA1 are expressed in trigeminal ganglion neurons (A, D, respectively). The somata of masseter afferents labeled by retrograde transport of Fast Blue (FB; arrows in B, and E) expressed TRPV1 (C) or TRPA1 (F).

Figure 2. Soma size distribution of TRPV1 and TRPA1 positive neurons in TG based on cell body area (μm²). The histograms were constructed from area measurements of 1360 TRPV1 and 465 TRPA1 positive neurons from 3 TG.

Figure 3.

Effects of masseteric capsaicin on immediate nociceptive responses (A,B) and ensuing changes in mechanical sensitivity (C,D). Effects of pre-treatment of the masseter with either vehicle or capsazepine on capsaicin-induced muscle nociception and mechanical hypersensitivity, along with the data from control groups, are also shown in E-H. Line graphs (A,C,E,G) show the time course of nociceptive hindpaw responses and changes in mechanical thresholds. Bar graphs (B,D,F,H) depict the total hindpaw responses and overall changes in mechanical sensitivity as AUC. Data in this figure and all subsequent figures are shown as mean $\pm$ SE. # denotes significant effect ($p < 0.05$) compared to the pre-injection baseline. ** denotes significant effect ($p < 0.01$) compared to vehicle.

Figure 4.

Effects of masseteric mustard oil on immediate nociceptive responses (A,B) and ensuing changes in mechanical sensitivity (C,D). Effects of pre-treatment of the masseter with either vehicle or AP18 on MO-induced muscle nociception and mechanical hypersensitivity, along with the data from control groups, are also shown in E-H. Line graphs (A,C,E,G) show the time course of nociceptive hindpaw responses and changes in mechanical thresholds. Bar graphs (B,D,F,H) depict the total hindpaw responses and overall changes in mechanical sensitivity as AUC.

Figure 5.

Effects of pre-treatment of the masseter with AMG9810 on capsaicin-induced muscle nociception and mechanical hypersensitivity, along with the data from control groups, are shown in A-D. Since both AMG9810 and capsazepine were dissolved in the same vehicle the data from vehicle control is the same data used in Fig 3. Effects of pre-treatment of the masseter with HC030031 on MO-induced muscle nociception and mechanical hypersensitivity, along with the data from control groups, are shown in E-F. Since both AP18 and HC030031 were dissolved in the same vehicle the data from vehicle control is the same data used in Fig 4.