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Role of TRPV1 and ASIC3 channels in experimental occlusal interference-induced hyperalgesia in rat masseter muscle

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Conflicts of interest
None declared.

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Abstract

Background: Masticatory muscle pain may occur following immediate occlusal alteration by dental treatment. The underlying mechanisms are poorly understood. Transient receptor potential vanilloid-1 (TRPV1) and acid-sensing ion channel-3 (ASIC3) mediate muscle hyperalgesia under various pathologic conditions. We have developed a rat model of experimental occlusal interference (EOI) that consistently induces mechanical hyperalgesia in jaw muscles. Whether TRPV1 and ASIC3 mediate this EOI-induced hyperalgesia is unknown.

Methods: Rat model of EOI-induced masseter hyperalgesia was established. Real-time polymerase chain reaction, Western blot and retrograde labelling combined with immunofluorescence were performed to evaluate the modulation of TRPV1 and ASIC3 expression in trigeminal ganglia (TGs) and masseter afferents of rats after EOI. The effects of intramuscular administration of TRPV1 and ASIC3 antagonists on the EOI-induced hyperalgesia in masseter muscle were examined.

Results: After EOI, gene expressions and protein levels of TRPV1 and ASIC3 in bilateral TGs were up-regulated. The percentage of ASIC3- (but not TRPV1-) positive neurons in masseter afferents increased after EOI. More small-sized and small to medium-sized masseter afferents expressed TRPV1 and ASIC3 separately following EOI. These changes peaked at day 7 and then returned to original status within 10 days after EOI. Intramuscular administration of the TRPV1 antagonist AMG-9810 partially reversed this mechanical hyperalgesia in masseter muscle. No improvement was exhibited after administration of the ASIC3 antagonist APETx2. Co-injection of AMG-9810 and APETx2 enhanced the effect of AMG-9810 administration alone.

Conclusions: Peripheral TRPV1 and ASIC3 contribute to the development of the EOI-induced mechanical hyperalgesia in masseter muscle.

1. Introduction

Masticatory muscle pain represents a considerable portion of craniofacial pain conditions (Palla and Farella, 2010). In dental practice, immediate alteration in occlusion by inappropriate dental intervention (iatrogenic occlusal interference) often results in discomfort in jaw muscles (Szentpétery et al., 2005; Le Bell et al., 2006), sometimes elicits persistent and intractable masticatory muscle pain (Hagag et al., 2000). The pathomechanisms underlying these clinical manifestations are elusive and available strategies are unpredictable (Svensson and Graven-Nielsen, 2001). We previously developed an experimental occlusal interference (EOI) model of rats that simulates this clinical condition and detected the

What's already known about this topic?
- Experimental occlusal interference (EOI) induces prolonged masseter mechanical hyperalgesia.
- Transient receptor potential vanilloid-1 (TRPV1) and acid-sensing ion channel-3 (ASIC3) are expressed in masseter afferents.

What does this study add?
- EOI modulates TRPV1 and ASIC3 expression in masseter afferents in the development of hyperalgesia.
- Blocking TRPV1 attenuates the EOI-induced masseter hyperalgesia. This anti-hyperalgesia effect can be enhanced by blocking TRPV1 and ASIC3 simultaneously.

mechanical hyperalgesia in bilateral masticatory muscles of rats which is aggravated for one week and sustained for more than 1 month (Cao et al., 2009). Further investigations demonstrated that this sustained hyperalgesia in jaw muscles is independent of muscle inflammation (Cao et al., 2010), but involved with central sensitization mechanisms (including neuronal hypersensitivity and glial activation in spinal trigeminal subnucleus caudalis in brain stem) (Cao et al., 2013). However, whether peripheral receptors located on the free nerve endings in masticatory muscle contribute to the EOI-induced hyperalgesia is unknown. Understanding peripheral receptor mechanisms may aid location of targets for pharmacologically alleviating masticatory muscle pain following occlusal interference as well as prevention of its transition to chronicity.

Two proton-sensitive receptor molecules, transient receptor potential vanilloid-1 (TRPV1) and acid-sensing ion channel-3 (ASIC3), have long been recognized to play vital roles in muscle pain and tenderness (Marchettini et al., 1996; McCleskey and Gold, 1999; Hoheisel et al., 2004; Mense, 2009). Animal (Light et al., 2008) and human (Pollak et al., 2014) studies have emphasized their physiological roles in the detection of metabolic change during muscle activity and the sensation of fatigue and pain. Plus, these two molecules are also involved in the development of mechanical hyperalgesia in muscle under various pathologic conditions such as inflammation (Sluka et al., 2007; Walder et al., 2011, 2012), overuse (Fujii et al., 2008) and chemotherapy (Hori et al., 2010). Besides, unilateral intramuscular administration of acidic saline evokes prolonged bilateral hyperalgesia (Sluka et al., 2001), suggesting their potential roles for the induction of chronic muscle pain. In trigeminal system, both TRPV1 and ASIC3 are expressed in masseter afferents in trigeminal ganglion (Connor et al., 2005). Administration of the TRPV1 agonist capsaicin significantly reduces mechanical threshold of masseter muscle (Arima et al., 2000; Ro et al., 2009). Pre-inhibition of TRPV1 suppresses αβmeATP-induced hyperalgesia in masseter muscle (Saloman et al., 2013). The role of ASIC3 in masticatory muscle pain is still undetermined. Masseter afferent neurons *in vitro* respond to pH change (Connor et al., 2005). However, injection of acidic saline in masseter fails to evoke hyperalgesia (Ambalavanar et al., 2007).

Heretofore, the role of TRPV1 and ASIC3 in the EOI-induced mechanical hyperalgesia in masseter muscle has not been explored. We hypothesized levels of TRPV1 and ASIC3 in masseter afferents increase after EOI and their antagonists relieve the EOI-induced mechanical hyperalgesia. Combination of real-time polymerase chain reaction (PCR), Western blot, immunofluorescence and behaviorial pharmacology was adopted to examine the hypothesis.

2. Materials and methods

2.1 Animals

One hundred and thirty eight male Sprague-Dawley rats (220-250 g; Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were used. Rats were housed under a 12-h light-dark cycle with food and water *ad libitum* in a temperature-controlled (23 °C) room. The experimental protocol was approved by the Animal Care and Use Committee of Peking University (Beijing, China).

2.2 Application of EOI

Details of the manufacture and application of a dental crown to induce experimental occlusal interference (EOI) has been described previously (Cao et al., 2009) and in the Supporting Information Methods S1. For sham-treated rats, the same procedure was performed but EOI was not applied. No intervention was given to naïve rats.

2.3 Behaviorial assessment of the mechanical sensitivity of masseter muscle

Mechanical hyperalgesia in masseter muscle was measured as the threshold of head withdrawal in

response to a mechanical stimulus over the belly region of masseter muscle. The detailed procedures are described previously (Cao et al., 2009), and in the Supporting Information Methods S2.

2.4 RNA extraction and real-time PCR

The detailed procedures are contained in the Supporting Information Methods S3.

2.5 Western blot analysis

Rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.). Bilateral TGs were acquired and homogenized in RIPA lysis buffer containing protease inhibitor cocktail (Beyotime Biotechnology, Shanghai, China). The homogenate was centrifuged at 4 °C for 20 min at 15000 g. The supernatant was collected and measured for protein concentration with a BCA protein assay kit (Beyotime Biotechnology). Forty μg total protein was loaded and separated electrophoretically in SDS-PAGE, then transferred to polyvinylidine difluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk and 0.1% Tween-20 in Tris-buffered saline at room temperature for 1 h, the membranes were incubated with primary antibody to TRPV1 or ASIC3 (1:1000, in blocking buffer, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C, followed by 2 h with horseradish peroxidase-conjugated secondary antibody (1:10000; Proteintech Group, Chicago, IL, USA). Anti-GAPDH (1:1000, Good Here Biotechnology, Hangzhou, China) was used as internal control. The membranes were developed in ECL solution (Beyotime Biotechnology) for 2 min and examined with a luminescent image analyzer (Fusion FX, VILBER LOURMAT, France). The intensity of immuno-positive bands was quantified with ImageJ 1.38 software (National Institutes of Health, Bethesda, MD, USA), normalized to the internal control, and expressed as fold changes of naïve rats.

2.6 Retrograde labelling of masseter afferents and immunofluorescence

Primary afferent neurons in TGs innervating masseter muscle were identified by retrograde labelling with 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Animals were anesthetized by isoflurane inhalation (5% induction, 2–3% maintenance). Masseter muscle was exposed carefully by cutting apart superficial skin and separating fascia tissue. DiI (2 mg/mL dissolved in dimethyl sulfoxide, DMSO) was injected slowly using a 25 μL microsyringe with a 30-G needle (5 μL for each site; five sites for each masseter muscle). To avoid tracer leakage to other tissues, the needle was left *in situ* for 2 min before its slow withdrawal. The injection site was covered with petroleum jelly and sutured (4.0 silk) carefully.

After 10 days, rats were perfused via a transcardial approach with 250 mL of body-temperature physiological saline (0.9%), followed by 300 mL ice-cold 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS; pH 7.4). TGs were dissected out and post-fixed in the same fixative at 4 °C overnight, then transferred to 30% sucrose for cryoprotection. Meanwhile, masseter muscles were also dissected out and the injection sites were examined to ensure tracer was confined to the masseter muscle. Tissue sections (thickness, 10 μm) of TGs were cut on a cryostat below −20 °C and thaw-mounted onto adhesive microscope slides (Citoglas, Beijing, China). Two of every ten consecutive sections were collected separately for immunofluorescence staining of TRPV1 and ASIC3. In total, five sections were included for each receptor per TG.

After blocking with 4% normal goat serum and 0.3% Triton-X 100 (Sigma-Aldrich) in PBS, sections were incubated separately with rabbit polyclonal primary antibody for TRPV1 (1:400 dilution; Santa Cruz Biotechnology) and ASIC3 (1:1000; Abcam, Cambridge, UK) for 2 days at 4 °C, followed by 90 min with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum (1:200; Zhong Shan Golden Bridge, Beijing, China) at room temperature. Primary antibody was omitted from selected sections to control for non-specific background staining. Stained sections were examined under a Fluorescence Microscope (BX51; Olympus, Tokyo, Japan). Digital images of DiI-labelled cells observed under two filters for DiI and FITC separately were produced using a charge-coupled device camera (DP71; Olympus) connected to the microscope.

Image analysis was performed with Image-Pro Plus v6.0 (Media Cybernetics, Rockville, MD, USA). Only the clearly labelled cells that present a discernable nucleus were included. The Number of DiI-labelled and double-labelled (DiI and FITC) cells in each section were counted. The perimeter of the soma of each double-labelled cell was traced manually and the cross-sectional area of each traced cell was calculated by the software. The size distributions of TRPV1- and ASIC3-positive masseter afferent neurons in each group were analysed. Labelled cells

were classified by size as small (<400 μm^2), medium (400–1000 μm^2) and large (>1000 μm^2) (Ichikawa and Sugimoto, 2001). Percentages of TRPV1- and ASIC3-immunoreactive neurons in afferent neurons innervating masseter muscles in TGs were calculated using the following formula:

TRPV1-/ASIC3-immunoreactive neurons in masseter afferent neurons in TGs = total number of double-labelled neurons in five sections × 100/total number of DiI-positive neurons in five sections.

The observer was blinded to the study protocol.

2.7 Intramuscular administration of antagonists

The effect of administration of antagonists on mechanical sensitivity in masseter muscle was examined 7 days after application of EOI (when muscle hyperalgesia was most severe). The TRPV1 antagonist AMG-9810 (Santa Cruz Biotechnology) in DMSO (0.1, 1 or 100 mmol/L; 50 μL for each masseter muscle) and ASIC3 antagonist APETx2 (Alomone Laboratories, West Jerusalem, Israel) in 0.9% saline (0.22 or 2.2 μmol/L; 50 μL for each masseter muscle) were injected. The dose of each antagonist used in this study had proven effective in other studies (Karczewski et al., 2010; Lee et al., 2012). Animals were anesthetized by isoflurane inhalation during injection at multiple sites in the right masseter muscle. Thresholds for bilateral head withdrawal were measured before as well as 30 and 60 min after antagonist administration. Effects of vehicles and combination of the two antagonists were investigated by injection in the same manner. Per cent reversal of mechanical hyperalgesia in masseter muscle was calculated according to the following formula:

$$\%\text{reversal} = (\text{post-injection threshold} - \text{pre-injection threshold}) \times 100/(\text{Baseline threshold} - \text{pre-injection threshold})$$

2.8 Statistical analyses

Statistical analyses were carried out using SPSS v20.0 (SPSS, Chicago, IL, USA). Data were tested for normality (Kolmogorov–Smirnov test) and equal variance. Data distributed normally with equal variance were analysed using Student's *t*-test, paired t-test, one-way or two-way analysis of variance (ANOVA) or repeated-measures ANOVA followed by a Bonferroni post hoc test where appropriate. These data were expressed as mean ± SEM. For the data not distributed normally or with unequal variance, Mann–Whitney *U* test or Kruskal–Wallis analysis of variance on ranks followed by Dunn's multiple comparison test was carried out alternatively. These data were represented with medians and interquartile range in figures. Value of $p < 0.05$ was considered significant.

3. Results

3.1 Protein levels of TRPV1 and ASIC3 in TGs after application of EOI

Protein levels of TRPV1 and ASIC3 in bilateral TGs of naïve rats ($n = 4$) and rats after application of EOI ($n = 4$ for each time point) or sham operation ($n = 3$ for each time point) for 3, 7, 10 and 14 days were examined. Compared with naïve rats, protein levels of TRPV1 in bilateral TGs upregulated at day 7, and had resumed to baseline level by day 14 after application of EOI (Kruskal–Wallis ANOVA on ranks followed by Dunn's test, $p < 0.05$). An increase trend of ASIC3 in bilateral TGs was observed at day 3 following EOI, which was significant at day 7, and had declined to baseline level by day 14 (Kruskal–Wallis ANOVA on ranks followed by Dunn's test, $p < 0.05$). No significant difference in TRPV1 or ASIC3 level in bilateral TGs was detected between naïve rats and rats received sham operation for 3, 7, 10 or 14 days (Fig. 1).

3.2 Retrograde tracing of masseter afferents and immunofluorescence

Since no overexpression of TRPV1 and ASIC3 was detected in sham-treated rats by real-time PCR (see Supporting Information Results S2) and Western blot, immunoreactivity of TRPV1 and ASIC3 in the masseter afferent sensory neurons in the TGs of rats that had undergone EOI for 3, 7, 10 and 14 days ($n = 4$ per time point) and naïve rats ($n = 4$) for baseline level was examined using retrograde tracing with the neuronal tracer DiI combined with immunofluorescence methods. Retrograde-labelled neurons were observed in TGs 10 days after intramuscular injection and limited to the mandibular portions of TGs. Total 12497 DiI-labelled neurons were counted in 40 TGs from 20 rats, ranging from 207 to 473 per ganglion (312.4 ± 9/TG). No difference in the number of labelled masseter afferents was demonstrated between right and left TGs as well as EOI groups and naive rats, suggesting the method that retrograde labelling with DiI injection is stable in respect of the side or group effect (two-way ANOVA, side effect, $F(1, 30) = 1.47$, $p = 0.2350$; group effect, $F(4, 30) = 0.55$, $p = 0.6974$). No signal was detected in negative control sections. In whole TG, neurons

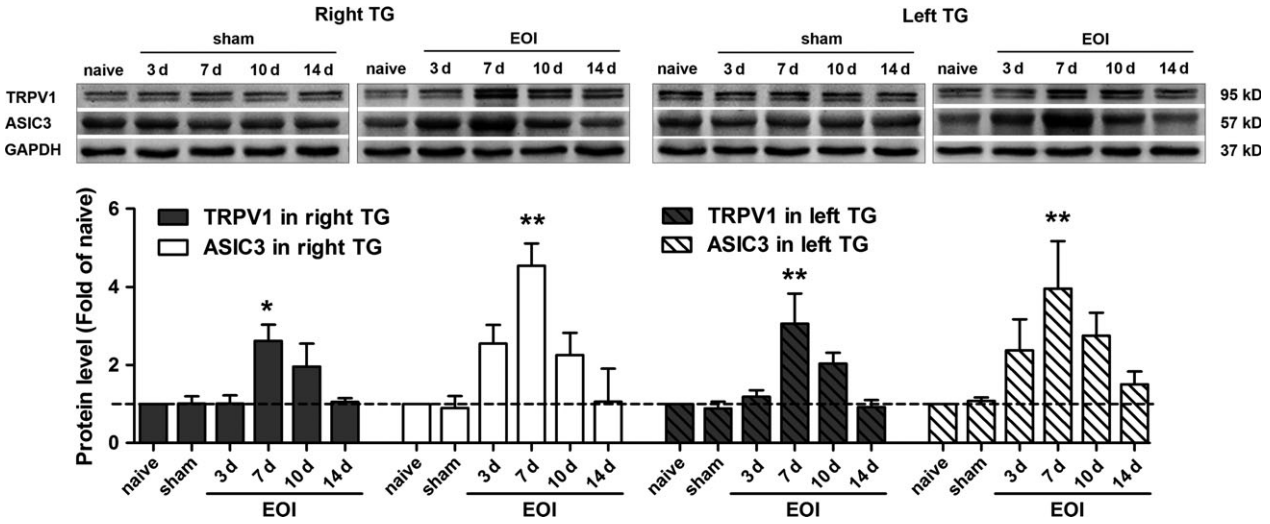

Figure 1 Protein levels of TRPV1 and ASIC3 in bilateral trigeminal ganglia (TGs) of naïve rats (n = 4), sham-treated rats for 3, 7, 10 and 14 days (n = 3 per time point) and rats that had undergone experimental occlusal interference (EOI) for 3, 7, 10 and 14 days (n = 4 per time point). Data were normalized using the internal control of GAPDH and the relative amounts to naïve rats were calculated. Examples of Western blot are shown on the top and the histograms illustrating the increased protein levels (fold changes) relative to naïve rats are shown in the bottom. Data of all sham-treated rats were combined since no difference among different time points after sham operation was detected. Data are expressed as median with interquartile range. *$p < 0.05$, **$p < 0.01$, compared with the naïve rats.

expressing TRPV1 or ASIC3 were distributed in all three divisions of TG. Based on the analysis of the merged pictures, DiI-labelled neurons containing TRPV1 or ASIC3 were detected in TGs (Fig. 2). Total 2209 TRPV1-positive and 3749 ASIC3-positive masseter afferent neurons were identified. TRPV1-immunoreactive neurons were predominately small to medium-sized cells and ASIC3-immunoreactive neurons consisted of small, medium and larger cells.

In naïve rats, an average of 38 ± 0.9% masseter afferent neurons in TG expressed TRPV1. No significant change in the per cent of TRPV1-positive masseter afferents was detected in TGs over the time course of the observation after EOI application [F (4, 30) = 0.88, p = 0.4857]. No significant difference in the per cent of TRPV1-positive masseter afferent neurons was found between right and left TGs [F (1, 30) = 1.82, p = 0.1874] (Figs 3A and B). An average of 50 ± 1% of masseter afferent neurons are ASIC3-positive in naïve rats. After application of EOI, there was a significant increase in the per cent of ASIC3-positive masseter afferents that started at day 3 (60 ± 0.8%), peaked at day 7 (68 ± 0.6%) and resumed from day 10 (60 ± 0.8%) to baseline level at day 14 (50 ± 0.7%) [F (4, 30) = 67.24, $p < 0.01$]. The upregulation of ASIC3 expression in masseter afferents exhibited identically in right and left TGs [F (1, 30) = 0.07, p = 0.7890] (Figs 4A and B).

Analysis of soma size of both TRPV1 and ASIC3-positive masseter afferent neurons revealed a left shit in the size frequency distribution after EOI application, which showed at day 3, pronounced at day 7, and resumed from day 10 to day 14, compared with naïve rats (Figs 3C–G and 4C–G). A significant decrease in the size of TRPV1-positive masseter afferent neurons in rats 7 days after EOI (median area = 421.2 μm^2, range 146.9–1142.0 μm^2) compared with naïve rats (median area = 470.8 μm^2, range 130.8–1286.0 μm^2) was detected (Kruskal–Wallis ANOVA on ranks followed by Dunn's test, $p < 0.01$). The size of ASIC3-positive masseter afferent neurons in rats with 3, 7 and 10 days of EOI was significantly smaller than that in naïve rats (3 day, median area = 640.8 μm^2, range 166.9–1995.0 μm^2; 7 day, median area = 564.6 μm^2, range 159.2–1762.0 μm^2; 10 day, median area = 706.2 μm^2, range 224.6–2042 μm^2; baseline, median area = 773.1 μm^2, range 271.5–3024.0 μm^2; Kruskal–Wallis ANOVA on ranks followed by Dunn's test, $p < 0.01$).

3.3 Antagonist effects on mechanical hyperalgesia induced by EOI

3.3.1 Effect of vehicle administration on mechanical hyperalgesia in masseter muscles

There was no significant difference in the head withdrawal threshold of masseter muscles on both sides in all rats 30 and 60 min after vehicle administration compared with pre-injection ($p > 0.05$) (Figs 5A and B).

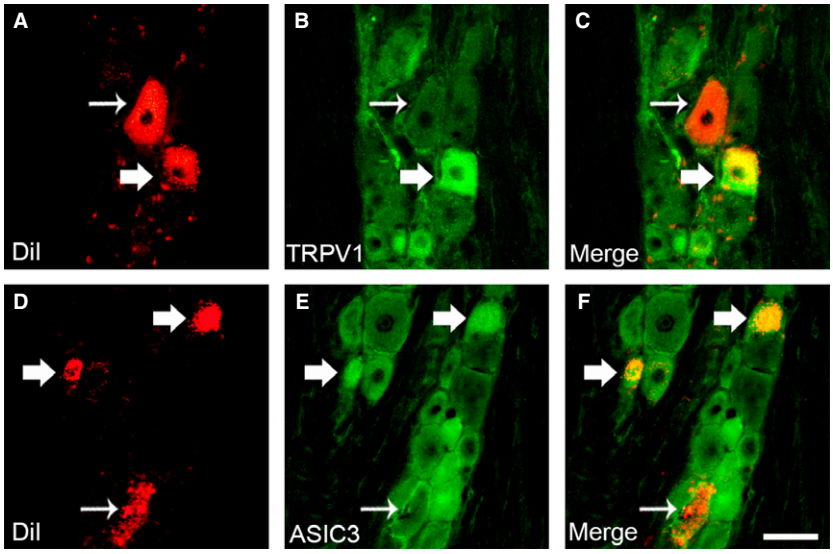

Figure 2 Immunofluorescence of the expression of TRPV1 and ASIC3 in masseter afferent neurons. Masseter afferent neurons in trigeminal ganglion were labelled from injection of DiI into the masseter muscle (A and D). Immunoreactivity for TRPV1 and ASIC3 of these neurons are demonstrated in B and E, also in the merged double-labelled picture C and F, respectively. Thin arrows indicate DiI-labelled neurons without expression of TRPV1 or ASIC3. Thick arrows indicate double-labelled neurons. Scale bar, 40 μm.

3.3.2 Effect of the TRPV1 antagonist AMG-9810 on mechanical hyperalgesia in masseter muscles

Compared with pre-administration, an increase in the head withdrawal threshold in the ipsilateral masseter muscle 30 min after AMG-9810 administration (repeat measures ANOVA, $p < 0.05$) was noted but disappeared after 60 min. No such effect was observed in the contralateral muscle. Three graded concentrations of AMG-9810 (1, 10 and 100 mmol/L) were administrated. A much stronger reversal of the EOI-induced hyperalgesia after 10 mmol/L AMG-9810 administration ($47 \pm 1.3\%$) was observed than that after 1 mmol/L AMG-9810 administration ($13 \pm 1.7\%$) (One-way ANOVA followed by Bonferroni post hoc test, $p < 0.01$), suggesting AMG-9810 dose-dependently attenuates the EOI-induced mechanical hyperalgesia in masseter muscle. No difference in the reverse effect between 100 mmol/L ($49 \pm 2.3\%$) and 10 mmol/L AMG-9810 ($47 \pm 1.3\%$) was noticed. The maximal effect of AMG-9810 was able to achieve about 50% reversal of the EOI-induced hyperalgesia in masseter muscle (Fig. 5C–E and H).

3.3.3 Effect of the ASIC3 antagonist APETx2 on mechanical hyperalgesia in masseter muscles

No significant difference was noted in bilateral mechanical sensitivity in masseter muscle 30 min and 60 min after two graded doses of APETx2 (0.22 and 2.2 μmol/L) administration compared with pre-administration ($p > 0.05$; Fig. 5F).

3.3.4 Effect of a combination of AMG-9810 and APETx2 on hyperalgesia in masseter muscle

To examine the synergistic effect of the two antagonists, a mixture of AMG-9810 (10 mmol/L) and APETx2 (2.2 μmol/L) was prepared and administrated in masseter muscle. Although there was no effect of APETx2 administration upon hyperalgesia in masseter muscle, combined injection of AMG-9810 and APETx2 showed stronger reverse effect on hyperalgesia in ipsilateral masseter muscle than the maximal effect of AMG-9810 administration alone ($64 \pm 1.5\%$ vs. $49 \pm 2.3\%$; $p < 0.01$) (Fig. 5I). The effect of combined administration on ipsilateral masseter muscle disappeared after 60 min and was not detected in the contralateral side (Fig. 5G).

4. Discussion

Regulation of TRPV1 or ASIC3 expression has been detected in spinal dorsal root ganglia under several kinds of pathologic conditions (Hudson et al., 2001; Amaya et al., 2003; Asai et al., 2005; Poirot et al., 2006; Fujii et al., 2008; Ikeuchi et al., 2009; Hori et al., 2010) as well as in trigeminal ganglia under facial inflammation (Pei et al., 2007). The constituent expressions of TRPV1 and ASIC3 in masseter afferents have been reported (Connor et al., 2005;

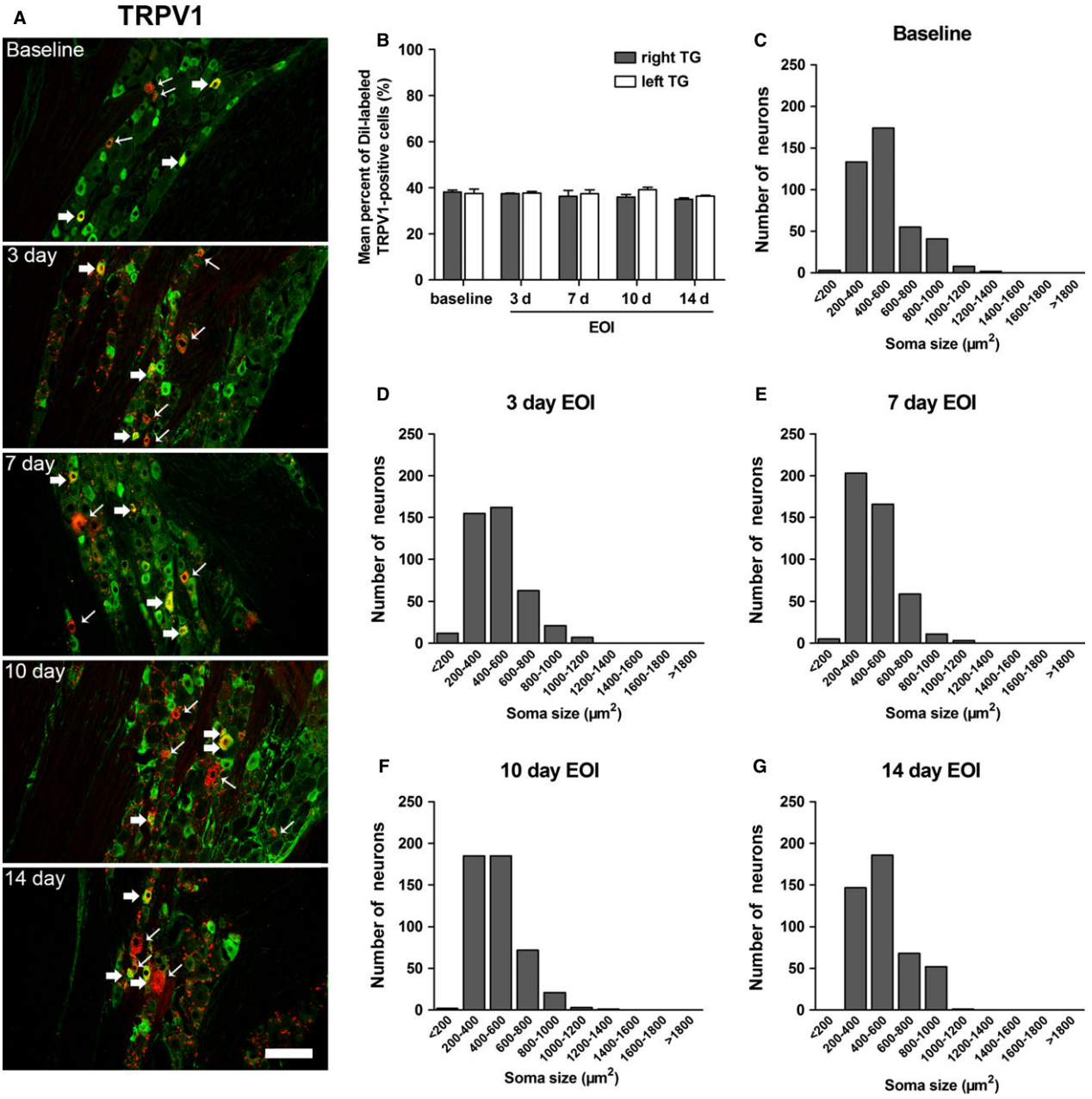

Figure 3 The expression of TRPV1 in masseter afferent neurons in trigeminal ganglia (TGs) of naive rats and rats with experimental occlusal interference (EOI) for 3, 7, 10 and 14 days. (A) Representative fluorescence photomicrographs showing TRPV1 immunoreactivity in masseter afferents and (B) mean percentage of TRPV1-positive neurons in DiI-labelled neurons in TGs of naive rats and rats with EOI for 3, 7, 10 and 14 days. Thin arrows indicate DiI-labelled neurons without expression of TRPV1. Thick arrows indicate double-labelled neurons. Scale bar, 100 μm. Error bars represent SEM. $n = 4$ in each group. (C–G) Size distribution of TRPV1-positive masseter afferent neurons in naive rats (417 neurons counted) and rats with EOI for 3, 7, 10 and 14 days (420, 447, 471 and 454 neurons counted, respectively).

Takeda et al., 2005; Ambalavanar et al., 2007; Ro et al., 2009). However, studies regarding the regulation of TRPV1 or ASIC3 expression underlying orofacial muscle pain are few. In this study, real-time PCR demonstrated transiently elevated levels of TRPV1 mRNA mainly at day 7, as well as ASIC3 mRNA at day 3 and more prominent at day 7 in TGs after EOI (Supporting Information Results S2), and Western blot exhibited transient increase in protein levels of TRPV1 and ASIC3 in TGs significantly at

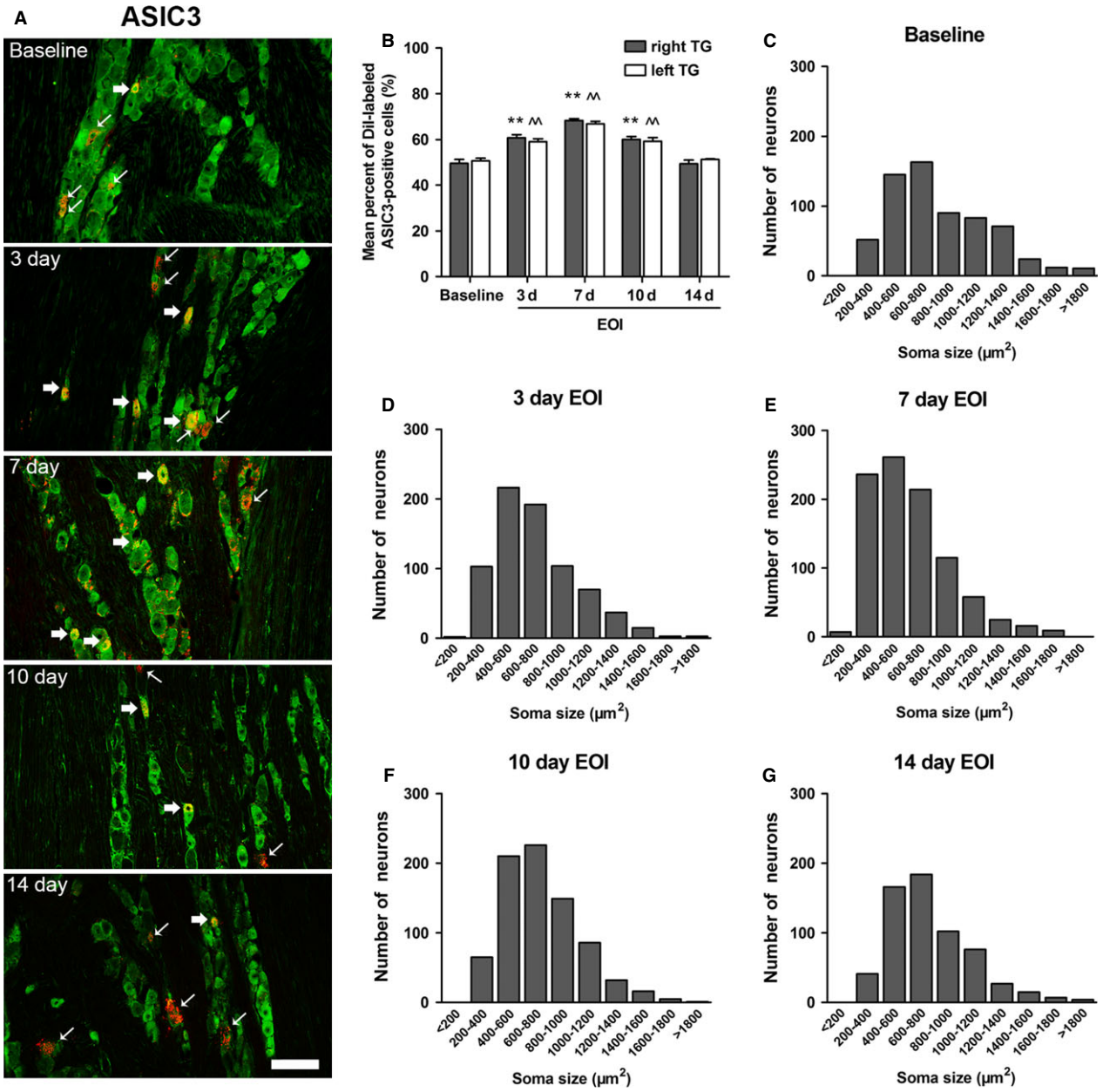

Figure 4 The expression of ASIC3 in masseter afferent neurons in trigeminal ganglia (TGs) of naive rats and rats with experimental occlusal interference (EOI) for 3, 7, 10 and 14 days. (A) Representative fluorescence photomicrographs showing the ASIC3 immunoreactivity in masseter afferents and (B) mean percentage of ASIC3-positive neurons in DiI-labelled neurons in TGs of naive rats and rats with EOI for 3, 7, 10 and 14 days. Thin arrows indicate DiI-labelled neurons without expression of ASIC3. Thick arrows indicate double-labelled neurons. Scale bar, 100 μm. **, ^^$p < 0.01$, compared with the naive rats. Error bars represent SEM. $n = 4$ in each group. (C–G) Size distribution of ASIC3-positive masseter afferent neurons in naive rats (651 neurons counted) and rats with EOI for 3, 7, 10 and 14 days (745, 941, 790 and 622 neurons counted, respectively).

day 7 after EOI, which temporally correlated with the most severe hyperalgesia in masseter muscle, suggesting potential roles of TRPV1 and ASIC3 in the EOI-induced mechanical hyperalgesia. As the results of real-time PCR and Western blot reveal their expressions in the whole TG, the expressions of TRPV1 and ASIC3 in masseter afferents in TG were further explored using retrograde labelling technique combining immunohistochemistry. We reported approximate 38% TRPV1-positive and 50% ASIC3-positive masseter afferents in naïve rats, comparable to other studies which reported the per cent of

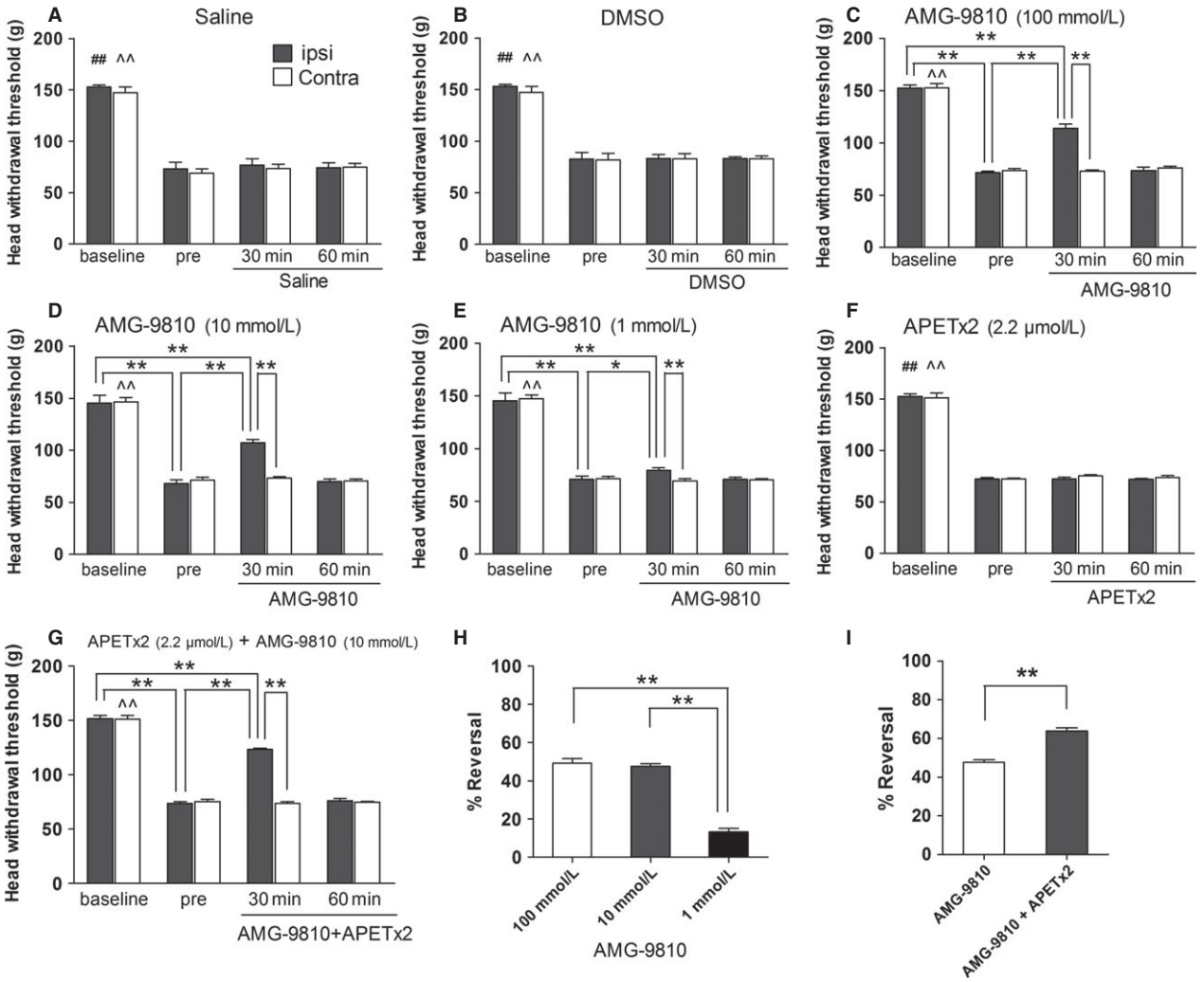

Figure 5 Effects of antagonists or vehicles on mechanical hyperalgesia induced by experimental occlusal interference (EOI) in masseter muscle. Head withdrawal thresholds of bilateral masseter muscles measured for three consecutive days before application of EOI were averaged and provided baseline data. Antagonists or vehicles were administrated in the right masseter muscle 7 days after application of EOI. Head withdrawal thresholds of bilateral masseter muscles were measured before as well as 30 and 60 min after intramuscular injection of (A) saline, (B) DMSO, (C) AMG-9810 (100 mmol/L), (D) AMG-9810 (10 mmol/L), (E) AMG-9810 (1 mmol/L), (F) APETx2 (2.2 μmol/L) and (G) APETx2 (2.2 μmol/L)+AMG-9810 (10 mmol/L) (n = 5 per group). Comparison of the effects of administration of graded concentrations of AMG-9810 (100, 10 or 1 mmol/L) as well as AMG-9810 (10 mmol/L) and combined injection of AMG-9810 (10 mmol/L) and APETx2 (2.2 μmol/L) on head withdrawal thresholds of the right masseter 30 min after injection 7 days after application of EOI is exhibited in (H) and (I), respectively. Formula for % reversal: % reversal = (post-injection threshold − pre-injection threshold) × 100/(Baseline threshold − pre-injection threshold). ipsi, ipsilateral masseter muscle of injection (right side); contra, contralateral masseter muscle of injection (left side); pre, pre-injection. ##$p < 0.01$, compared with other data on the ipsilateral side. ^^$p < 0.01$, compared with other data on the contralateral side. *$p < 0.05$ and **$p < 0.01$. Error bars represent SEM.

TRPV1-positive masseter afferents ranges from 24% to 37% (Takeda et al., 2005; Ro et al., 2009), and ASIC3-positive masseter afferents from 49% to 64% (Ambalavanar et al., 2007; Zhang et al., 2008). After EOI application, no change was observed in the per cent of TRPV1-positive masseter afferents. But a left shift of their size distribution occurred at day 3, 7 and 10 (significantly at day 7), reflecting more TRPV1 are expressed in small-sized cells. The discrepancy of the results of TRPV1 expression detected in TG (levels of mRNA and protein) and that in masseter afferents (per cent of TRPV1-positive neuron) at day 7 may be explained by the possibility that the total number of cells containing TRPV1 did not increase but the gross expression of TRPV1 in these cells elevated. Besides, upregulated TRPV1 expression

in TG may also occur in other peripheral afferents in TG (such as afferents innervating tooth-pulp or jaw joint) which may be affected by EOI (reviewed by Clark et al., 1999; Xie et al., 2013) and have shown to express TRPV1 (Ichikawa and Sugimoto, 2001; Ichikawa et al., 2004). For the limitation of our technique, further studies that can quantify the TRPV1 expression in specific subsets of TGs may be required to fully illustrate these data. Although not all the data of TRPV1 are positive, the present results still suggest that transient modulation of TRPV1 expression occurs in masseter afferent after EOI. The per cent of ASIC3-positive masseter afferents was elevated by day 3, peaked at day 7 and sustained till day 10 after EOI. Analysis of size distribution exhibited that upregulated ASIC3 are expressed mainly in small to medium-sized cells at these time points. The time course of the regulation of TRPV1 or ASIC3 expression in these small or small to medium-sized cells was consistent with that of their mRNA or protein alteration in the whole TG. Since unmyelinated and thin-myelinated muscle afferent fibres (group IV and III) account for nociception (Graven-Nielsen and Mense, 2001), the elevated expressions of TRPV1 and ASIC3 particularly in small or medium-sized cells are probably present in these nociceptors. The regulations of TRPV1 and ASIC3 expression may play a role in sensitizing the primary afferent neurons (probably nociceptors) in response to the interstitial pH change during masticatory muscle activity and contributing to the development of mechanical hyperalgesia in masseter muscle after EOI. Interestingly, the transient overexpressions of TRPV1 and ASIC3 were observed in bilateral TGs after EOI. Also, no difference of the percentage of TRPV1- or ASIC3-positive masseter afferents between right and left TGs was detected. These data suggest the same neuroplastic change occurs in bilateral TGs which may contribute to the bilateral hyperalgesia caused by unilateral EOI, and bilateral intervention targeting the peripheral receptors may be necessary for clinically alleviating this condition.

To further confirm the functions of TRPV1 and ASIC3 in the EOI-induced mechanical hyperalgesia in masseter muscle, the effects of local administration of their specific antagonists were examined. The TRPV1 antagonist AMG-9810 can dose-dependently attenuate the mechanical hyperalgesia in masseter after EOI, strongly implying the direct role of TRPV1 in this hyperalgesia. No improvement was detected in the contralateral muscle, suggesting local administration of the antagonist did not exhibit systemic action. The maximal effect of blocking TRPV1 can achieve about 50% reversal of the EOI-induced masseter hyperalgesia. We did not examine the effect of AMG-9810 on mechanical sensitivity in masseter of normal rats, but negative results have been reported by researchers who administrated AMG-9810 in an identical way to us (Lee et al., 2012; Saloman et al., 2013), suggesting the regularly expressed TRPV1 in masseter afferents may not play a functional role in pressure pain sensation in normal status. Administration of the ASIC3 specific inhibitor APETx2 at two graded doses did not exhibit improvement. The doses of APETx2 we used have been shown to effectively reverse acid-induced hyperalgesia in gastrocnemius muscle of rats (Karczewski et al., 2010). Interestingly, simultaneous administration of AMG-9810 and APETx2 presented a stronger reversal than the maximal effect of AMG-9810, showing a synergistic effect of these two blockers. These results may be explained by the possibility that ASIC3 did not mediate the EOI-induced hyperalgesia directly but could reinforce the function of TRPV1 via some unknown mechanisms, such as forming a kind of functional complex by interacting with TRPV1. Then, the intensified effect of this functional complex could be inhibited much more effectively by blocking TRPV1 and ASIC3 simultaneously than just blocking one of them. Similar speculations regarding the interaction of TRPV1 and ASIC3 receptors have also been proposed by Ambalavanar and Dessem (2009) and Light et al. (2008). The synergistic action of blockers of TRPV1 and ASIC3 has been reported in the muscle afferents responsible for exercise pressor reflexes in rats (Li et al., 2004; Gao et al., 2006). Through double staining immunofluorescence combined with retrograde tracing (See Supporting Information), we demonstrated the co-expression of TRPV1 and ASIC3 in masseter afferents, consistent with the results of a previous study using *in vitro* electrophysiological recordings (Connor et al., 2005). Besides, the percentage of TRPV1 and ASIC3 co-expressed masseter afferents increased at day 7 after EOI compared with baseline level, which further supports our speculation for the synergistic anti-hyperalgesia effect of blockers of TRPV1 and ASIC3. However, more studies are required to verify this speculation and to discover the detailed mechanisms underlying the interaction of these two molecules.

One consideration regarding to the roles of peripheral TRPV1 and ASIC3 in the EOI-induced hyperalgesia in masseter muscle is that their expressions were mainly modulated within 10 days after application of

EOI, but the mechanical hyperalgesia in masseter muscle can last at least for 1 month. The prolonged hyperalgesia may involve the central mechanisms. The activation of glia in spinal trigeminal nucleus following EOI can persist for 1 month (Cao et al., 2013). Glia cells have been suggested to play a powerful role in nociception (Watkins and Maier, 2003). Thus, the peripheral TRPV1 and ASIC3 may be involved more in the development than the maintenance of the EOI-induced hyperalgesia.

In summary, we demonstrated the modulation of expressions of TRPV1 and ASIC3 in masseter afferents following EOI application, and verified the direct role of TRPV1 and indirect role of ASIC3 in the EOI-induced mechanical hyperalgesia in masseter muscle. Simultaneous inhibition of TRPV1 and ASIC3 can alleviate this hyperalgesia more effectively. Our findings could be important to understand the peripheral mechanisms of occlusal interference-induced masticatory muscle pain and the development of novel drug treatment strategy. Timely and effective intervention against muscle pain means a lot to prevent the development of chronic pain.

Author contributions

X.X.X. performed the most parts of experiments and data analyses, drafted the article and approved the final version of this manuscript. Y.C. and Q.F.X. conceived and designed the experiments, helped perform the analyses and revised the article and approved the final version of this manuscript. Y.C. also helped to perform the experiments. T.T.D. helped to perform the experiments and data analyses and approved the final version of this manuscript. K.Y.F. and Y.L. contributed to data interpretation and revised the article and approved the final version of this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Methods S1. Application of EOI.
Methods S2. Behavioral assessment of the mechanical sensitivity of masseter muscle.
Method S3. RNA extraction and real-time PCR.
Method S4. Co-expression of TRPV1 and ASIC3 in masseter afferents in TG after EOI.
Results S1. Mechanical hyperalgesia in masseter muscles after application of EOI.
Results S2. Gene expressions of TRPV1 and ASIC3 in TGs after application of EOI.
Results S3. Co-expression of TRPV1 and ASIC3 in masseter afferents in TGs after EOI.
Discussion S1. Hyperalgesia in masseter muscle in the rat model of EOI.
Figure S1. Time course of the head withdrawal thresholds caused by mechanical stimulation of masseter muscle on each side of rats with or without EOI.
Figure S2. Gene expression of TRPV1 and ASIC3 in bilateral TGs of naive rats, sham-treated rats and EOI rats.
Figure S3. Co-expression of TRPV1 and ASIC3 in masseter afferents in TGs of naive rats and EOI rats.