Upregulation of the Purinergic Receptor Subtype P2X3 in the Trigeminal Ganglion Is Involved in Orofacial Pain Induced by Occlusal Interference in Rats

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Aims: To evaluate whether the purinergic receptor subtype P2X3 (P2X3R) in trigeminal ganglion (TG) neurons is involved in hyperalgesia of the temporomandibular joints (TMJs) and masseter muscles associated with placement of an occlusal interference. **Methods:** Forty-five rats were randomized into five groups (ie, for days 1, 3, 7, 14, or 28; nine rats per group). Six rats from each group were chosen to receive the occlusal interference, and the remaining three rats were sham-treated controls. On days 1, 3, 7, 14, and 28 after placement of the occlusal interference, the mechanical pain threshold (MPT) to stimulation of the TMJs or masseter muscles was examined using von Frey filaments. Seven days after the occlusal interference placement, changes in MPT were tested after administration of the P2X3R antagonist A-317491 into the TMJs and masseter muscles (60 μg/site) in six rats. The expression of P2X3R in the TGs was investigated by immunohistochemistry and quantitative polymerase chain reaction (qPCR). Retrograde tracing was combined with immunofluorescence to identify TMJ and masseter muscle afferent neurons in the TGs of six premature rats. **Results:** The TMJ and masseter muscle MPTs were decreased after placement of the occlusal interference, and the P2X3R antagonist reversed the mechanical hyperalgesia that was caused by the occlusal interference placement. The frequency of P2X3R-immunoreactive cells increased in small-sized neurons in the TG after occlusal interference. By contrast, there was no increase in medium-sized TG neurons. P2X3R mRNA increased on day 3. Retrograde tracing indicated that the TMJ and masseter muscle afferent neurons in the TG expressed P2X3R. **Conclusion:** Upregulated P2X3R expression in the TG may contribute to orofacial pain development induced by an occlusal interference. P2X3R may be a therapeutic target for chronic TMJ or masseter muscle pain.
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Keywords: *masseter muscle, occlusal interference, orofacial hyperalgesia, P2X3 receptor, temporomandibular joint*

Up to 10% of the population suffer from temporomandibular disorders (TMD) at least once in their lifetime.[1] Persistent pain in the region of the temporomandibular joint (TMJ) and/or related masticatory muscles is the main symptom reported by patients with TMD. Many studies have shown a multifactorial etiology of TMD, and improper occlusion has been suggested as a risk factor.[2] However, an occlusal etiology of TMD remains controversial, and many studies have not found sufficient evidence in support of this etiology.[1,3]

According to several studies and reviews, the hyperalgesia of the TMJ and related masticatory muscles is thought to result from sensitization of trigeminal sensory neurons.[4] Recently, ATP has been recognized as an important neurostimulator and has been widely studied in pain processing.[5–7] ATP acts on purinergic P2X and P2Y receptors, and the P2X3 subtype (P2X3R), which is one of seven P2X receptor subtypes, can be activated by ATP and has been shown to play a role in nociceptive transmission.[8]

In the trigeminal ganglion (TG), P2X3R is expressed on both small and medium-sized neurons,[9] and has been reported on trigeminal sensory neurons innervating the TMJ and masseter muscle.[10–12] In addition,

Table 1 Number of Pain-Related Responses and the Corresponding Scores

Pressure (g)	Pain-related responses (n)	Score
100	0	0
100	1	0.2
100	2	0.4
100	3	0.6
100	4	0.8
100	5	1.0
60	1	1.2
60	2	1.4
60	3	1.6
60	4	1.8
60	5	2.0
26	1	2.2
26	2	2.4
26	3	2.6
26	4	2.8
26	5	3.0

there is evidence that supports P2X3R involvement in nociceptive transmission in animals with an inflamed TMJ.[11,13] However, little is known about the involvement of P2X3R in nociceptive transmission of occlusion-related TMD.

In the present study, an animal model of occlusal interference was established to evaluate whether P2X3R in TG neurons is involved in hyperalgesia of the TMJs and masseter muscles associated with placement of an occlusal interference.

Materials and Methods

Animals

The study used 51 male Wistar rats weighing 200 to 250 g, as well as six premature rats (3 weeks old; they were selected to identify TMJ and masseter muscle afferent neurons in the TGs). Animals were kept in a temperature-controlled room (25°C) and exposed to a 12-hour light-dark cycle with food and water ad libitum. The study was conducted after approval from the animal ethics committee of Shandong University (No. ECAESDUSM 2012). Efforts were taken to minimize the number of experimental animals used in this study, and their suffering.

Occlusal Interference Animal Model

Forty-five rats were randomized into five groups (nine rats per group for days 1, 3, 7, 14, and 28). Six rats in each group were randomly selected to receive the occlusal interference, and the remaining three rats were sham-treated controls. Modified crowns made from Co-Cr alloy were applied to the mandibular first molars of the six experimental rats in each group. The crowns had a thickness of 1 mm at the occlusal surface and were designed to cover the occlusal, buccal, lingual, and mesial surfaces. Furthermore, the difference between crown-applied rats and sham rats in the interocclusal distance was 1 mm.[14] The six experimental animals in each group were anesthetized with an intraperitoneal injection of 10% chloral hydrate (30 mL/kg), and the crowns were bonded to the mandibular right first molars with dental resin cement (Super-Bond C&B, Sun Medical). The three sham-treated rats were anesthetized and their mouth was gently forced open for about 5 minutes (as for experimental rats), but no crown was placed.

Mechanical Hyperalgesia

The mechanical pain threshold (MPT) was assessed using von Frey filaments with silicone rubber pellets of 1 mm in diameter at the tip (Semmes-Weinstein monofilaments, Stoelting) to avoid cutaneous pain during the testing,[14] which was similar to that described by Ren et al.[15,16] Rats were allowed to acclimatize to their surroundings for 15 minutes and were observed not to withdraw or flick their heads away from the tip of the filaments. During the test, two orofacial areas were tested: the masseter muscle region, at a site 10 mm inferior to the central point of the line between the orbit and the tragus, and the TMJ region, which was positioned at a site 5 mm ahead of the tragus.

A graded series of three von Frey filaments was used during the test, which produced a bending force of 26, 60, and 100 g. Force was applied with the filaments, which were placed perpendicular to the surfaces of the testing points, and each rat was stimulated on both sides. The stimulation always began by using the filament that produced the lowest force. Each stimulus filament was applied five times onto the testing points for 3 to 5 seconds with intervals of 2 to 3 minutes. Both left and right sides were tested in each rat. A rapid head flinching or an attack reaction (biting or grabbing the filament) was considered to represent a pain-related response, as it was most consistent and easy to characterize. MPT was defined as scores that were assessed by the appearance of the pain-related response (Table 1), and higher scores meant a lower MPT.[17]

The analysis of the nociceptive behaviors was made by an investigator who was blind to the side of crown application. The rats underwent pilot testing using von Frey filaments 7 days before the formal testing. The MPT scores decreased with time to a final stabilized score. Therefore, the scores of MPT on day 7 were considered as the baseline. The MPT was subsequently tested at days 1, 3, 7, 14, and 28 after crown application.

Peripheral Administration of P2X3R Antagonist

On day 7 after crown application, the effects of the peripheral administration of the selective nonnucleotide P2X3R antagonist A-317491 (Santa Cruz Biotechnology) on the MPT were studied in the six crown-treated rats in the remaining groups (ie, days 14 and 28); the three sham-operated rats were controls. After anesthesia by ether inhalation, A-317491 was injected in three random rats to both sides of the TMJs and masseter muscles, and at a dose of 60 μg (1 M, 60 μL) per injection site. The dose of antagonist used in this study has previously been shown to be effective.[18] Since the antagonist was dissolved in isotonic saline, the remaining three rats received the vehicle alone as control. Before administration of either antagonist or isotonic saline, animals were tested for their baseline MPT values. Then, MPTs were tested 180 minutes after administration. The observer was also blind to the treatment received.

Tissue Preparation

On each of days 1, 3, 7, 14, and 28 after placement of the occlusal interference, three rats in each group were overdosed with chloral hydrate, and transcardially perfused with heparinized saline followed by a cold fixative containing 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.2). Their scalps were skinned, and the brains and muscles around the TMJs were removed. TGs, TMJs, and masseter muscles were clearly identified bilaterally, immediately dissected after perfusion, and immersed in the same fixative overnight at 4°C. After dehydration and paraffin embedding, the TGs and masseter muscles were sectioned in the horizontal plane along the long axis of the TG or muscle fiber at a thickness of 4 μm. After decalcification in 10% EDTA, the TMJs were dehydrated, embedded in paraffin, and sectioned on the sagittal plane at a thickness of 4 μm. For analysis, five sections per TG were chosen for each rat.

The other three rats in each group were also anesthetized with an intraperitoneal injection of chloral hydrate. The TGs were dissected on both sides, rinsed with cold sterile saline solution, and stored at −80°C for quantitative polymerase chain reaction (PCR) assay. Total RNA was extracted from the TGs by using TRIZOL (TaKaRa Bio) and used (1 μL) for complementary DNA (cDNA) synthesis using the first-strand cDNA synthesis kit (TaKaRa Bio), according to the manufacturer's recommendations.

Histology of TMJs and Masseter Muscles

To visualize the pathologic changes after the crown application, sections of the TMJs and masseter muscles were stained using hematoxylin and eosin (Solarbio).

Immunohistochemistry

After routine deparaffinization and rehydration, the sections underwent antigen retrieval in 0.125% trypsin-EDTA (Solarbio) for 20 minutes at 37°C. HistostainTM-Plus kits (ZSGB-Bio) were used according to the manufacturer's recommendations. After incubation in goat serum, sections were incubated with the rabbit anti-P2X3R polyclonal antibody (Bioss) at a dilution of 1:800 in 0.01 M PBS overnight at 4°C. After rinsing with 0.01 M PBS, the sections were exposed to a solution of peroxidase-conjugated avidin-biotin complex (1:100; Vector Laboratories) for 20 minutes at 37°C. Sections were then visualized with 0.1% 3,3'-diaminobenzidine dihydrochloride (DAB) (ZSGB-Bio). The digital images were captured using a microscopy digital camera system (Olympus) and were analyzed using Image-Pro Plus 6.0 software. The neurons that were stained two-fold more intense than the average background were considered immunoreactive (IR) for P2X3R. No specific labeling was observed in the absence of the primary antibody. The neurons were classified into three groups according to the neuronal size: small neurons (< 400 μm^2), medium neurons (> 400 and < 1,000 μm^2), and large neurons (> 1,000 μm^2).[19] The number of P2X3R-IR neurons in each animal was calculated using the equation 100 × total number of P2X3R-IR neurons in five sections of the TG/total number of neurons in five sections of the TG. The frequency of small-sized and medium-sized neurons was also calculated.

Quantitative PCR

Quantitative PCR was performed using the LightCycler 480 system (Roche) and the FastStart DNA Masterplus SYBR Green kit (Roche). The reaction product was quantified with the Relative Quantification tool (LightCycler Software 4, Roche Diagnostics), with rat β-actin as the reference gene. The sequences of the P2X3R primers were: forward 5'-CAG GGC ACC TCT GTC TTT GTC-3' and reverse 5'-TCA GAC ACA CAG CGG TAC TT-3'; The sequences of the β-actin primers were: forward 5'-CAT TGC TGA CAG GAT GCA GAA G and reverse 5'-GAG CCA ATC CAC ACA GAG T-3'. Real-time PCR was performed with initial denaturation at 95°C for 10 minutes, followed by 30 cycles of 95°C for 5 seconds and 60°C for 30 seconds.

Identification of the TMJ and Masseter Muscle Afferent Neurons in the TGs

The TMJ and masseter afferent neurons in the TGs were identified by using retrograde labeling with hydroxy-stilbamidine (Fluoro-gold, [FG], Biotium), as described in a previous study,[11] but using premature rats (3 weeks old) instead of naïve Lewis rats as used by Shinoda et al because they are easier to operate

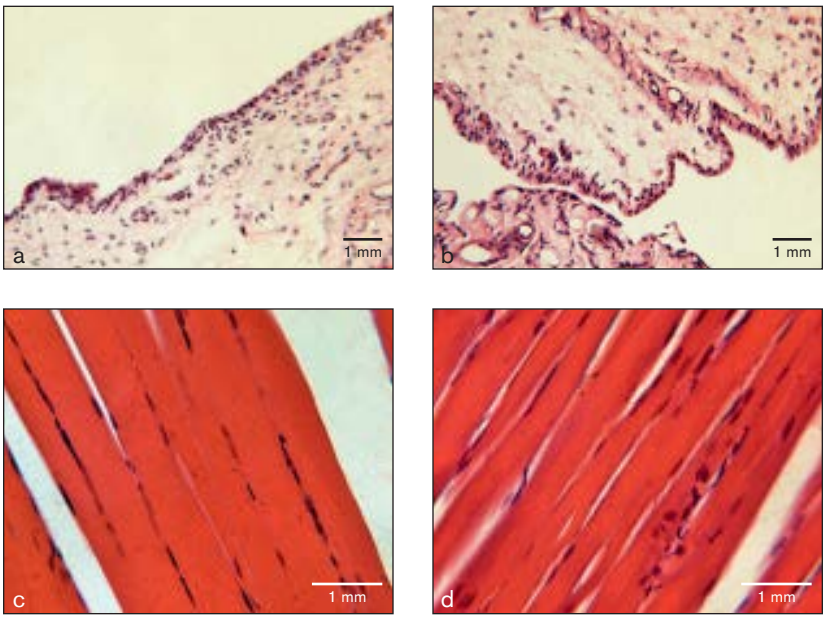


Fig 1 Histology of the TMJ and masseter muscles in hematoxylin-eosin stained sections. The synovial membrane of the TMJ in the **(a)** sham-treated controls and **(b)** experimental rats is shown. Masseter muscle tissue in **(c)** sham-treated controls and **(d)** experimental rats is also shown. Scale bar = 50 μm. Arrows indicate myofibrils with central nuclei.

on. After anesthesia with diethyl ether, three premature rats were injected with a 2% solution of FG in saline into the upper joint cavity of the TMJs (2 μL per TMJ), and three premature rats received injections into the masseter muscle region (2 μL per masseter).

One week after the FG injection, all animals were anesthetized and then perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.01 M PBS. The TGs were quickly dissected, post-fixed in the same fixative for 4 hours, and cryoprotected by infiltration with 30% sucrose solution overnight at 4°C. The TGs were cut into 7-μm sections on a cryostat along the long axis of the ganglion, and five sections in each rat were chosen for immunohistochemistry. All sections were incubated with diluted rabbit anti-P2X3R polyclonal antibody (1:100) overnight at room temperature. Sections were then reacted with the fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Bioss) at a concentration of 1:100 in PBS, and coverslipped in mounting medium (Beyotime). A negative control test was obtained by omitting the primary antibody, and no specific labeling was observed. The positive neurons (FG-labeled, FITC-labeled, or double-labeled) were examined under a fluorescence microscope (Olympus) equipped with a digital camera and using appropriate filters.

Statistical Analyses

Continuous data are expressed as mean ± SEM. The Kruskal-Wallis test was applied to compare three or more groups; if there was a difference, the Mann-Whitney *U* test was used for comparing two groups, which was followed by the LSD post hoc test. Statistical analysis was performed using the SPSS v 17.0 statistical analysis software (IBM). *P* values < .05 were considered significant.

Results

Histology of TMJs and Masseter Muscles

In the experimental model, the TMJs and masseter muscles of rats did not show significant inflammatory cellular infiltration, but they showed an increase in synovial folds in the TMJs and hyperplasia of some synoviocytes. There was vasodilation in the synovial lamina propria (Fig 1b). Disarrays of myofibers with central nuclei were observed in the masseter muscles (Fig 1d). These phenomena are consistent with those reported in a previous study.[20] These abnormalities were not observed in the sham-treated controls (Fig 1a).

Nociceptive Behaviors

TMJ and masseter muscle hyperalgesia was induced on both ipsilateral and contralateral sides following application of the occlusal interference. The TMJ and masseter muscle hyperalgesia induced by the occlusal interference began on day 1, peaked on day 7, and persisted at least until 28 days (Fig 2). In addition, the reduction of the MPTs on both ipsilateral and contralateral sides showed no significant difference. No significant change in the MPTs of the sham-treated group was observed during the experimental period compared to baseline.

Effect of P2X3R Antagonist on MPT

On day 7 after crown application, at which time the rats showed significant mechanical hyperalgesia, the injection into the TMJs and masseter muscles of A-317491, as compared with isotonic saline treatment alone, reversed the bilateral mechanical hyperalgesia in the TMJs and masseter muscles (Fig 3).

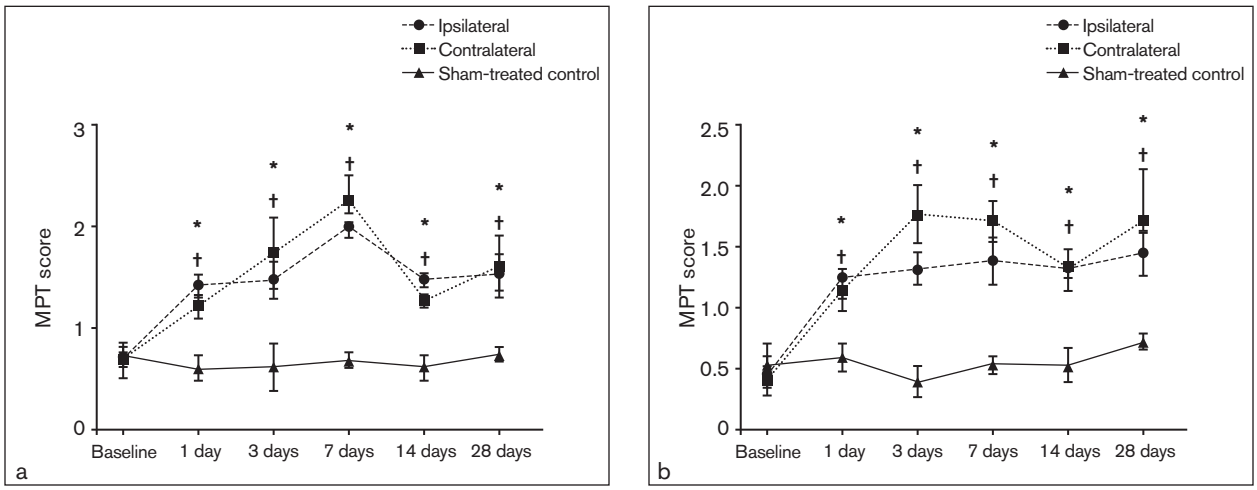


Fig 2 Time course of the mechanical pain threshold (MPT) in the TMJs and masseter muscles after experimental occlusal interference. The occlusal interference increased pain-related responses (ie, reduced the MPTs) evoked by stimulation of the **(a)** masseter muscles on both sides and **(b)** TMJs. This effect started on day 1 after crown application, peaked on day 7, and persisted for the duration of the study period. Data are shown as mean ± SEM. **P* < .05 vs the ipsilateral side and sham-treated controls; †*P* < .05 vs the contralateral side and sham-treated controls.

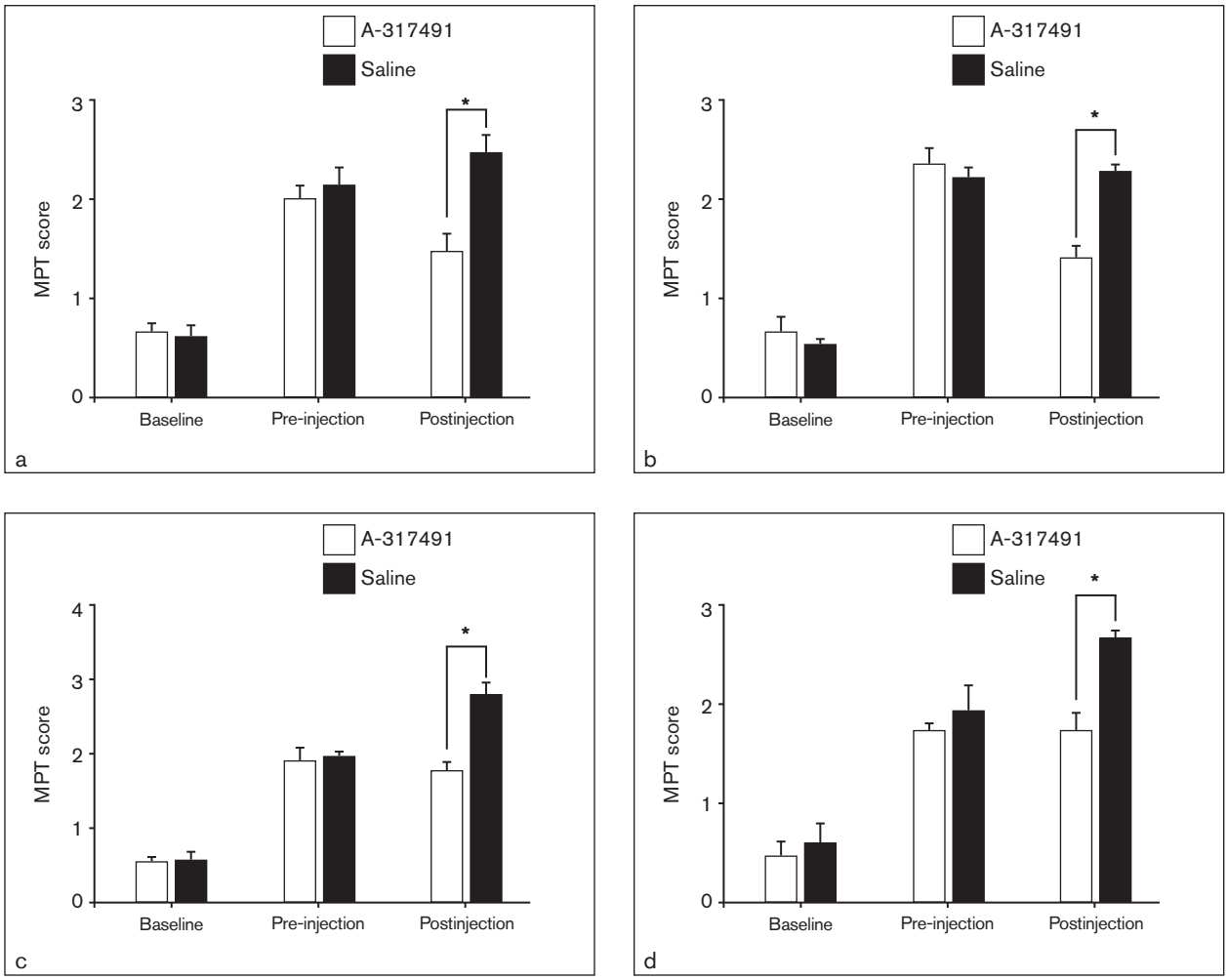


Fig 3 Comparison of the mechanical pain threshold (MPT) in TMJs and masseter muscles after injection of A-317491 or isotonic saline on day 7 after crown application. **(a)** Right masseter muscles. **(b)** Left masseter muscles. **(c)** Right TMJ. **(d)** Left TMJ. Data are shown as mean ± SEM. **P* < .05 vs isotonic saline.

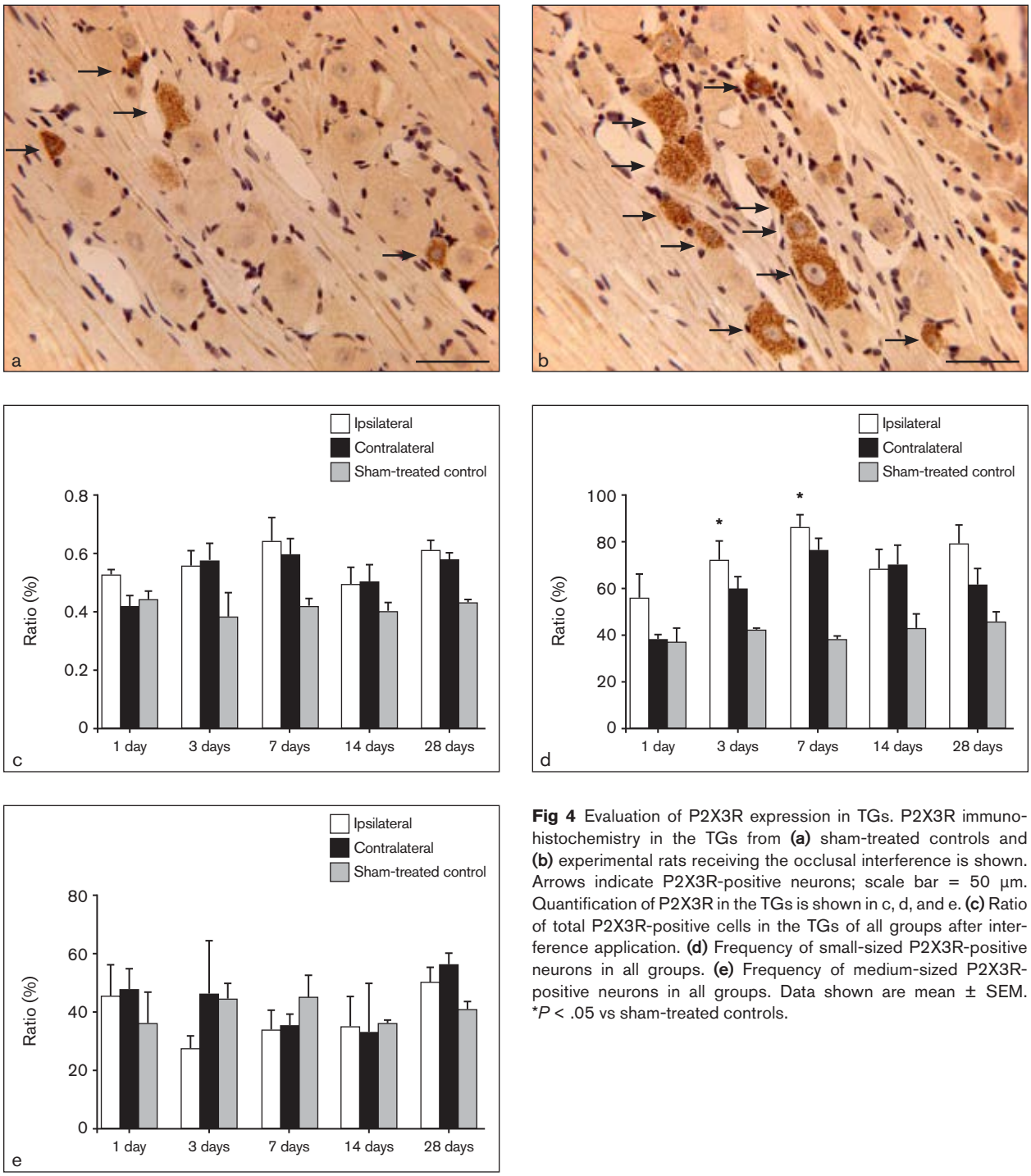


Fig 4 Evaluation of P2X3R expression in TGs. P2X3R immunohistochemistry in the TGs from **(a)** sham-treated controls and **(b)** experimental rats receiving the occlusal interference is shown. Arrows indicate P2X3R-positive neurons; scale bar = 50 μm. Quantification of P2X3R in the TGs is shown in c, d, and e. **(c)** Ratio of total P2X3R-positive cells in the TGs of all groups after interference application. **(d)** Frequency of small-sized P2X3R-positive neurons in all groups. **(e)** Frequency of medium-sized P2X3R-positive neurons in all groups. Data shown are mean ± SEM. **P* < .05 vs sham-treated controls.

P2X3R-IR Cells in TGs

The P2X3R-IR cells could be distinguished from the non-IR cells in the TGs (Figs 4a and 4b). The IR cells were abundant among the small- and medium-sized neurons, and few were observed in the large-sized neurons in the TGs. After interference application, the percentage of P2X3R-IR cells was not significantly increased in the ipsilateral and contralateral TGs as compared with sham-treated rats (*P* > .05; Fig 4c). However, compared with sham-treated rats, an increase in the small-sized P2X3R-IR neurons was observed in the ipsilateral TGs on days 3 and 7, peaking on day 7 (*P* < .05; Fig 4d), although the proportion of the medium-sized P2X3R-IR neurons in the ipsilateral and contralateral TGs was not significantly higher as compared with the sham-treated rats (*P* > .05)

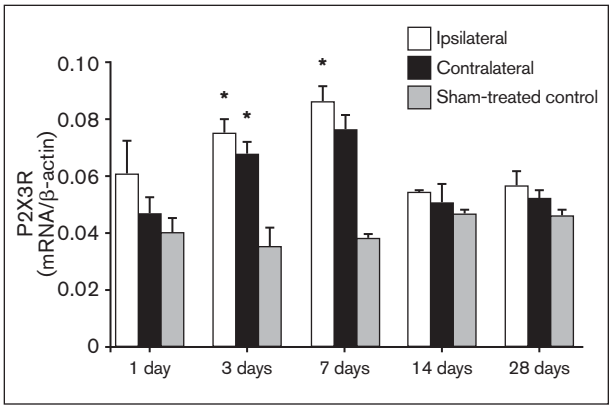
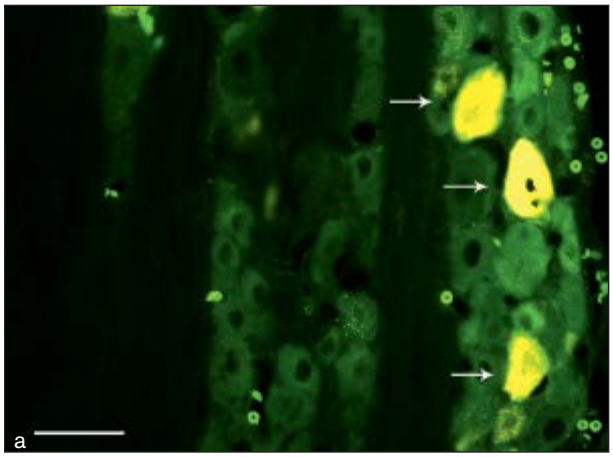
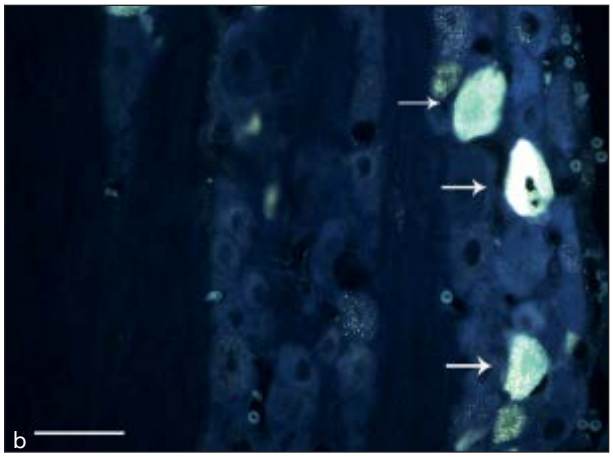


Fig 5 *(right)* Expression of mRNA P2X3R in the TGs by quantitative PCR. On day 3, the expression of P2X3R had increased in the group receiving the occlusal interference; on day 7, the increase was still evident in the ipsilateral TGs of this group. Data shown are mean ± SEM. *$P < .05$ vs sham-treated controls.

Fig 6 *(below)* Retrograde staining of P2X3R in TG neurons innervating the masseter and TMJ in the TGs of premature rats. **(a)** P2X3R-positive cells. **(b)** FG-labeled neurons. Arrows indicate double-labeled neurons. Scale bar = 50 μm.





(Fig 4e). Also, in all experimental groups, there was no significant difference between the ipsilateral and contralateral TGs in the frequency of small-sized and medium-sized neurons.

P2X3R mRNA Expression

The expression of P2X3R mRNA in the TGs was examined by quantitative PCR. Compared with the sham-treated rats, the expression of P2X3R mRNA in the group receiving the occlusal interference was significantly increased on day 3, without any difference being observed between the ipsilateral and contralateral TGs in the experimental group (Fig 5).

Retrograde Neuronal Tracing

Retrogradely labeled cells were detected in the TGs at 1 week after the injection of the fluorescent neuronal tracer into the TMJs and masseter muscles.[11] FG-positive cells with P2X3R-IR were present in the TGs (Fig 6). In the TGs, 14.4 ± 2.0% of the neurons innervated the TMJs (FG-positive cells), and 18.1 ± 2.5% of the neurons innervated the masseter muscles. Furthermore, 44.1 ± 7.0% of the neurons innervating the TMJs and 48.5 ± 5.4% of the neurons innervating the masseter muscles manifested P2X3R-IR expression.

Discussion

The aim of this study was to evaluate whether P2X3R in TG neurons is involved in TMJ and masseter muscle hyperalgesia associated with placement of an occlusal interference. Results showed that the MPT of the bilateral TMJs and masseter muscles was decreased after occlusal interference and persisted for the entire experimental period of 28 days. The P2X3R antagonist A317491 reversed the hyperalgesia induced by the occlusal interference.

The frequency of small-sized P2X3R-IR neurons in the TGs increased after placement of the occlusal interference. By contrast, there was no increase found for the medium-sized P2X3R-IR neurons. Quantitative PCR showed that the P2X3R mRNA increased on day 3 after the occlusal interference placement. Retrograde tracing confirmed that the afferent neurons innervating TMJs or masseter muscles in the TGs expressed P2X3R.

In previous studies, experiments on animal models, involving injection of algesic agents in the region of the TMJ and related masseter muscle and excessive jaw movements, have been used to study hyperalgesia of the TMJs and masseter muscles.[11–13,21] However, injection of algesic agents could not imitate most clinical conditions that are known to induce pain in the TMJ and masseter muscle. According to the symptoms, the pain of the TMJ region and masseter muscle is frequently reported after an improper or pathologic occlusion.[2] Therefore, an animal model using an artificial occlusal interference is closer to the clinical reality. Despite the fact that the relationship between occlusal interference and masseter muscle pain remains controversial, some studies conducted in animals and humans have suggested that this relationship does exist.[22] Some previous studies have identified occlusion as a major risk factor for TMJ pain,[22–27] while other studies have not.[28–30] However, many factors are involved in TMJ and masseter muscle pain, thus making it very difficult to determine the exact role of occlusion in this type of pain. Nevertheless, a number of studies using different approaches suggest that occlusal interference is involved in hyperalgesia, and that TGs are involved.[22]

Compared with several other methods of occlusal alteration,[31–33] crown application as used in the present study has the advantage of allowing quantitative measurements, long-term retention, and harmless effects to the pulp. In the present study, the modified crowns were bonded to the mandibular first molars of rats, thus establishing an animal model of occlusal interference. The bilateral hyperalgesia of the TMJs and masseter muscles was rapidly induced after the crown application, and lasted for at least 4 weeks. These results are consistent with a clinical study showing that facial pain appeared in volunteers within 3 days after induction of a unilateral occlusal highspot, and remained for at least 6 days.[34] Results of the present study strongly suggest that occlusal interference can directly cause chronic TMJ and masseter muscle pain in an experimental animal model. Combined with some of the previous clinical findings, the findings support an occlusal etiology of TMD.

Recent studies that focused on purinergic receptors have explored pain processing, especially with respect to P2X receptors. Seven P2X receptor subtypes (ie, P2X1 to 7) have been cloned,[35] and all P2X receptor subtypes (with the exception of P2X7) are expressed in neurons that are located in sensory ganglia (including TG) and the primary afferent terminal region in areas such as the spinal dorsal horn and the trigeminal subnucleus caudalis.[36,37] Several studies have emphasized the role played by the P2X3R in nociceptive signaling. Indeed, Staikopoulos et al[9] have shown that about half the neurons in the TGs express the P2X2 receptor or P2X3R. Hsieh et al[38] observed that chemically induced sensory neuropathy was associated with enhanced expression of P2X3R. The P2X3R have also been found to play a role in dental, TMJ, and masseter muscle pain associated with orofacial inflammation.[11] Hu et al[39] have shown that P2X2R and P2X3R play a role in the initiation and maintenance of central sensitization in the trigeminal subnucleus caudalis and subnucleus oralis after application of mustard oil to the dental pulp. Chiang et al[40] and Jennings et al[41] have also shown that endogenous ATP is involved in this process. However, until the present study, no studies have reported on the relationship between the P2X3R and occlusion-related orofacial pain.

In the present study, it was demonstrated that the P2X3R in TGs is involved in the mechanical hyperalgesia of the TMJs and masseter muscles which was induced by the occlusal interference, and that the P2X3R antagonist A-317491 significantly reduced TMJ and masseter muscle hyperalgesia, consistent with previous findings with other antagonists of P2X3R.[40] P2X3R was predominantly expressed in the small-sized and medium-sized TG neurons, in agreement with previous studies.[11,19] After application of the occlusal interference, there were no observable changes in the total number of P2X3R-IR neurons and medium-sized P2X3R-IR neurons, but the proportion of P2X3R-IR small-sized neurons increased on day 3 and peaked on day 7, in association with the occurrence of hyperalgesia in the TMJs and masseter muscles. The expression of P2X3R mRNA was also markedly increased on day 3 after the occlusal interference. The reason the mRNA levels reached a peak that was seen earlier than that of the P2X3R-IR small-sized neurons might be due to the time delay for the production of proteins after mRNA synthesis. These results indicate that the hyperalgesia induced by the occlusal interference may be initially influenced by enhanced expression of P2X3R, and the small-sized neurons in the TGs may possibly participate in development of the hyperalgesia. Furthermore, the results of retrograde tracing demonstrated that almost half of the neurons innervating the TMJs and masseter muscles showed P2X3R-IR. In addition, the administration of the P2X3R antagonist A-317491 significantly attenuated TMJ and masseter muscle hyperalgesia that was associated with the application of the interference.

The present study was not without its limitations. The study was performed in animals, and therefore further studies are necessary before P2X3R antagonists might be considered for use in human subjects presenting with hyperalgesia of the TMJ and masseter muscles. In addition, the present study was not

designed to assess the mechanisms of P2X3R expression in the TGs, and further studies are necessary to address this.

Conclusions

The findings of this study suggest that P2X3R might play an important role in nociceptive transmission induced by an occlusal interference and might represent a new clinically relevant therapeutic target.

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References

1. LeResche L. Epidemiology of temporomandibular disorders: Implications for the investigation of etiologic factors. Crit Rev Oral Biol Med 1997;8:291–305.
2. Kirveskari P, Alanen P, Jämsä T. Association between craniomandibular disorders and occlusal interferences in children. J Prosthet Dent 1992;67:692–696.
3. Pullinger AG, Seligman DA, Gornbein JA. A multiple logistic regression analysis of the risk and relative odds of temporomandibular disorders as a function of common occlusal features. J Dent Res 1993;72:968–979.
4. Jennings EA, Williams MC, Staikopoulos V, Ivanusic JJ. Neurobiology of temporomandibular joint pain: Therapeutic implications. Semin Orthod 2012;18:63–72.
5. Chizh BA, Illes P. P2X receptors and nociception. Pharmacol Rev 2001;53:553–568.
6. Bleehen T, Keele CA. Observations on the algogenic actions of adenosine compounds on the human blister base preparation. Pain 1977;3:367–377.
7. Hamilton SG, Warburton J, Bhattacharjee A, Ward J, McMahon SB. ATP in human skin elicits a dose-related pain response which is potentiated under conditions of hyperalgesia. Brain 2000; 123:1238–1246.
8. North RA. P2X3 receptors and peripheral pain mechanisms. J Physiol 2004;554:301–308.
9. Staikopoulos V, Sessle BJ, Furness JB, Jennings EA. Localization of P2X2 and P2X3 receptors in rat trigeminal ganglion neurons. Neuroscience 2007;144:208–216.
10. Ichikawa H, Fukunaga T, Jin HW, Fujita M, Takano-Yamamoto T, Sugimoto T. VR1-, VRL-1- and P2X3 receptor-immunoreactive innervation of the rat temporomandibular joint. Brain Res 2004; 1008:131–136.
11. Shinoda M, Ozaki N, Asai H, Nagamine K, Sugiura Y. Changes in P2X3 receptor expression in the trigeminal ganglion following monoarthritis of the temporomandibular joint in rats. Pain 2005;116:42–51.
12. Shinoda M, Ozaki N, Sugiura Y. Involvement of ATP and its receptors on nociception in rat model of masseter muscle pain. Pain 2008;134:148–157.
13. Teixeira JM, Oliveira MC, Nociti FH Jr, et al. Involvement of temporomandibular joint P2X3 and P2X2/3 receptors in carrageenan-induced inflammatory hyperalgesia in rats. Eur J Pharmacol 2010;645:79–85.
14. Cao Y, Xie QF, Li K, Light AR, Fu KY. Experimental occlusal interference induces long-term masticatory muscle hyperalgesia in rats. Pain 2009;144:287–293.
15. Ogawa A, Ren K, Tsuboi Y, Morimoto T, Sato T, Iwata K. A new model of experimental parotitis in rats and its implication for trigeminal nociception. Exp Brain Res 2003;152:307–316.
16. Ren K. An improved method for assessing mechanical allodynia in the rat. Physiol Behav 1999;67:711–716.
17. Liu XD, Wang XD, Sun L, Zhang M, Fu KY. An animal model of masticatory muscle hyperalgesia induced by experimental individual molar movement in rats. Chin J Conserv Dent 2005;15: 542–545.
18. Wu G, Whiteside GT, Lee G, et al. A-317491, a selective P2X3/P2X2/3 receptor antagonist, reverses inflammatory mechanical hyperalgesia through action at peripheral receptors in rats. Eur J Pharmacol 2004;504:45–53.
19. Hwang IK, Lee HY, Yoo KY, et al. Chronological alterations of P2X3 receptor expression in the trigeminal ganglion after ischaemic insult in the Mongolian gerbil. Anat Histol Embryol 2004; 33:220–224.
20. Nishide N, Baba S, Hori N, Nishikawa H. Histological study of rat masseter muscle following experimental occlusal alteration. J Oral Rehabil 2001;28:294–298.
21. Schmidt-Hansen PT, Svensson P, Jensen TS, Graven-Nielsen T, Bach FW. Patterns of experimentally induced pain in pericranial muscles. Cephalalgia 2006;26:568–577.
22. Xie Q, Li X, Xu X. The difficult relationship between occlusal interferences and temporomandibular disorder—insights from animal and human experimental studies. J Oral Rehabil 2013; 40:279–295.
23. Ekberg E, Vallon D, Nilner M. The efficacy of appliance therapy in patients with temporomandibular disorders of mainly myogenous origin. A randomized, controlled, short-term trial. J Orofac Pain 2003;17:133–139.
24. Kurita H, Ohtsuka A, Kurashina K, Kopp S. A study of factors for successful splint capture of anteriorly displaced temporomandibular joint disc with disc repositioning appliance. J Oral Rehabil 2001;28:651–657.
25. De Boever JA, Carlsson GE, Klineberg IJ. Need for occlusal therapy and prosthodontic treatment in the management of temporomandibular disorders. Part II: Tooth loss and prosthodontic treatment. J Oral Rehabil 2000;27:647–659.
26. Sadowsky C, Theisen TA, Sakols EI. Orthodontic treatment and temporomandibular joint sounds—A longitudinal study. Am J Orthod Dentofacial Orthop 1991;99:441–447.
27. Egermark I, Blomqvist JE, Cromvik U, Isaksson S. Temporomandibular dysfunction in patients treated with orthodontics in combination with orthognathic surgery. Eur J Orthod 2000; 22:537–544.
28. Le Resche L, Truelove EL, Dworkin SF. Temporomandibular disorders: A survey of dentists' knowledge and beliefs. J Am Dent Assoc 1993;124:90–94,97–106.
29. Glaros AG, Glass EG, McLaughlin L. Knowledge and beliefs of dentists regarding temporomandibular disorders and chronic pain. J Orofac Pain 1994;8:216–222.
30. Arbree NS, Campbell SD, Renner RP, Goldstein GR. A survey of temporomandibular disorder conducted by the Greater New York Academy of Prosthodontics. J Prosthet Dent 1995; 74:512–516.

31. Stahl SS, Miller SC, Goldsmith ED. The influence of occlusal trauma and protein deprivation on the response of periapical tissues following pulpal exposures in rats. Oral Surg Oral Med Oral Pathol 1958;11:536–540.
32. Akagawa Y, Nikai H, Tsuru H. Histologic changes in rat masticatory muscles subsequent to experimental increase of the occlusal vertical dimension. J Prosthet Dent 1983;50:725–732.
33. Chen J, Zhang J, Zhao Y, et al. Hyperalgesia in response to traumatic occlusion and GFAP expression in rat parabrachial [correction of parabranchial] nucleus: Modulation with fluorocitrate. Cell Tissue Res 2007;329:231–237.
34. Li J, Jiang T, Feng H, Wang K, Zhang Z, Ishikawa T. The electromyographic activity of masseter and anterior temporalis during orofacial symptoms induced by experimental occlusal highspot. J Oral Rehabil 2008;35:79–87.
35. North RA. Molecular physiology of P2X receptors. Physiol Rev 2002;82:1013–1067.
36. Burnstock G. P2X receptors in sensory neurones. Br J Anaesth 2000;84:476–488.
37. Khakh BS. Molecular physiology of P2X receptors and ATP signalling at synapses. Nat Rev Neurosci 2001;2:165–174.
38. Hsieh YL, Chiang H, Lue JH, Hsieh ST. P2X3-mediated peripheral sensitization of neuropathic pain in resiniferatoxin-induced neuropathy. Exp Neurol 2012;235:316–325.
39. Hu B, Chiang CY, Hu JW, Dostrovsky JO, Sessle BJ. P2X receptors in trigeminal subnucleus caudalis modulate central sensitization in trigeminal subnucleus oralis. J Neurophysiol 2002;88:1614–1624.
40. Chiang CY, Zhang S, Xie YF, et al. Endogenous ATP involvement in mustard-oil-induced central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). J Neurophysiol 2005;94:1751–1760.
41. Jennings EA, Christie MJ, Sessle BJ. ATP potentiates neurotransmission in the rat trigeminal subnucleus caudalis. Neuroreport 2006;17:1507–1510.