Short communication

Tooth injury increases expression of the cold sensitive TRP channel TRPA1 in trigeminal neurons

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1. Introduction

The suppression of pain is of major clinical concern for a variety of conditions caused by disease or injury to tissues, including those within the oral cavity. Pain associated with tooth decay or trauma is frequently associated with an increased sensitivity to heat, cold, and other environmental stimuli. The dental pulp, the vital component of teeth, is densely innervated by free nerve endings which originate from neurons in the trigeminal ganglia (TG).1,2 Nerve endings within the pulp are equipped with a variety of ion channels, receptors and neuropeptides known to modulate nociception.3–6 TG neurons are therefore important cellular components in pain perception in the orofacial region. There is evidence that tooth injury is associated with increased expression of nociceptive ion channels in rodent TG neurons, suggesting that activation of pain pathways can induce global changes in trigeminal expression of nociceptive molecules7,8; however, a detailed understanding of the molecules and mechanisms involved in pain associated with tooth injury is lacking.

Recent studies have shown that temperature-sensitive channels of the transient receptor potential (TRP) family play a major role in sensing hot and cold temperatures and in pain perception associated with temperature.5,9 TRPV1 is a capsaicin-sensitive channel that is activated by elevated (>42°C) temperature.10 TRPM8 and TRPA1 channels are responsive to reduced temperatures when expressed in heterologous cells (<25°C and <18°C, respectively), and are selectively

## Article Info

- **Keywords:** Trigeminal ganglion, Pain, TRPA1 channel, Dental pulp, Western blot

## Abstract

Objective: Transient receptor potential (TRP) channels, a family of structurally related proteins have been implicated in the sensation of pain and hyperalgesia caused by exogenous and endogenous agonists, as well as touch, pH, and temperature. The objective of this study was to determine the effects of tooth injury on the expression of the cold sensitive channel TRPA1, in the trigeminal ganglion, the primary source of sensory and nociceptive innervation of teeth.

Design: We analyzed TRPA1 expression in a rodent model of tooth injury, by Western blot analyses of proteins extracted from trigeminal ganglia.

Results: We found that TRPA1 was selectively increased in trigeminal ganglia innervating injured teeth when compared to TRPA1 expression in trigeminal ganglia innervating healthy teeth.

Conclusions: Our results provide the first evidence of increased expression of a cold-sensitive TRP channel in trigeminal ganglia after pulp exposure, and are consistent with the possibility that increased expression and function of TRPA1 in trigeminal neurons contributes to hyperalgesia and allodynia following tooth injury.

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0003–9969/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2011.06.014
responsive to ligands such as menthol, and icilin.11–13 In contrast, the role of TRPA1 in cold sensation is less clear. Cellular and behavioural analyses of TRPA1-deficient mice showed that TRPA1 is not essential for acute responses to cold14; however, suppression of TRPA1 by antisense nucleotides reduces cold allodynia in rat models of inflammation and nerve injury.15 Other results also support the idea that TRPM8 is important for acute cold sensation under normal physiological conditions and that TRPA1 is a mediator of cold hypersensitivity in pathological conditions associated with inflammation.16,17

In addition to a role in noxious cold sensation, there is increasing evidence in support of a role for TRPA1 channels in a variety of nociceptive pathways. TRPA1 channels are activated by environmental irritants, oxidative stress, inflammatory peptides, and mechanical stress.18–21 The sensitivity of TRPA1 to tetrahydrocannabinol (THC) and morphine indicates that there may be other endogenous modulators of TRPA1 function.22,23 TRPA1-deficient mice exhibit pronounced deficits in bradykinin-evoked nociceptor excitation and pain hypersensitivity,24,25 suggesting that TRPA1 function is an important component of signalling pathways by which inflammatory mediators produce pain and hyperalgesia. There is further evidence that the bradykinin-induced inflammatory response occurs by sensitization of TRPA1 via phospholipase C and PKA mediated intracellular signalling pathways.26,27 Interestingly, bradykinin-induced hypersensitivity to heat, which is diminished in TRPV1-deficient mice, is also absent in TRPA1-deficient mice, indicating that TRPA1 and TRPV1 are interdependently regulated.28 Similarly, TRPA1 response to mustard oil, a TRPA1 agonist, is diminished in trigeminal neurons from TRPV1-deficient mice compared to controls.29 Together, the results suggest that in sensory neurons co-expressing TRPA1 and TRPV1, the activities of both channels are modulated via direct or indirect interactions between them. The exact nature of this interdependence remains to be investigated.

In humans, tooth injuries that expose the dentine and pulp generally produce pain, and are associated with an enhanced intensity of pain sensation or with the abnormal perception of pain from non-noxious stimuli. There is evidence that nociceptive ion channel expression increases in pulpal axons, and in cell bodies of TG after tooth injury.4,7,29 One explanation for the increased hypersensitivity to cold temperatures is that the density of functional TRP ion channels in the membranes of nociceptive neurons is increased after injury, thereby sensitizing the injured tooth to a variety of noxious stimuli. In rodent models of nerve injury, cold allodynia was associated with increased expression of TRPM8 in nociceptive afferent neurons30,31; however, TRPM8 expression was decreased in human teeth with irreversible pulpitis and cold hyperalgesia.32 Cultured rodent dental sensory afferents express transcripts encoding the cold-sensing TRPM8 and TRPA1 channels and are activated by changes in temperature and by thermo-TRP channel ligands such as menthol, and icilin.3 Electrophysiological, immunohistochemical, and single-cell RT-PCR evidence suggests that dental primary afferents have electrophysiological characteristics of nociceptors and express several nociceptor-specific ion channels, including the temperature-sensitive TRPV1, TRPA1, and TRPM8 channels.33 Changes in expression levels, activity or regulation of TRPA1 channels could therefore contribute significantly to tooth pain. Here, we show that TRPA1 channel proteins are transiently increased in TG isolated from rats subjected to a clinically relevant model of tooth injury compared to TG innervating uninjured teeth.

2. Materials and methods

All surgical and experimental procedures on animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with Federal guidelines.

2.1. Pulp exposure

Adult male Sprague-Dawley rats (~250 g; Hilltop Lab Animals) were anaesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). The mouth was held open with a micro-dissecting retractor and the mesial cusps of the left, mandibular first and second molars were prepared with a high-speed handpiece and a 1/4 round bur (Brasseler, Savannah, GA) until the pulp chamber was exposed. Verification of pulp exposure was made by tactile ‘give’ of tooth structure and the visualization of blood. Experimental (pulp-exposed) animals and controls were allowed to recover with softened food ad libitum. In one group of rats (n = 4), pulp exposures were performed on the first and second left molars. The left (injured side) and right (uninjured side) TG from these animals were isolated and stored at −80 °C on day 4 after pulp injury. In a second group of rats (n = 12), at 1, 4 and 7 days after pulp exposure, experimental animals (n = 3) and a control were anaesthetized with pentobarbital (80 mg/kg), and decapitated. Rats used as controls in this group received anaesthesia, but no pulp exposure. Left and right TG from each animal were isolated and stored at −80 °C.

2.2. Preparation of protein extracts

Individual trigeminal ganglia were rinsed, and homogenized in a buffered saline solution (20 mM Tris pH 7.4, 150 mM NaCl) containing a protease-inhibitor cocktail (Thermoscientific), using a Biomasher device (Daigger). Membrane proteins were extracted using the Pierce MEM-PER membrane protein extraction kit and prepared for gel electrophoresis using the Pierce SDS-PAGE sample preparation kit according to the manufacturer’s directions (Thermoscientific). An aliquot of each sample was saved for protein assays and the remainder was diluted in Laemmli sample buffer for electrophoresis and Western blotting using standard techniques. Briefly, membrane extracts from individual TG were separated on either 10% or 4–15% gradient gels by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BioRad), blocked with 1% casein in TBS-T (20 mM Tris–HCl, 500 mM NaCl, 0.1%Tween 20, pH 7.5) for 2 h at room temperature, and incubated overnight at 4 °C with a rabbit anti-TRPA1 antibody (Alomone Labs, 1:100 dilution). Blots were washed with TBS-T, and incubated with HRP-labelled donkey anti-rabbit secondary antibody (GE Healthcare, 1:1000 dilution) in TBS-T for 1 h at room temperature. After
multiple washes in TBS-T, membranes were treated with ECL reagent (Amersham Pharmacia Biotech) for detection of bound antibodies, and exposed to X-ray film. For incubations with preadsorbed antibody, 32 µg of TRPA1 antibody were incubated for 1 h at room temperature with 32 µg of the synthetic peptide (Alomone Labs) used to generate the antibody. The mixture was then centrifuged and the supernatant used at 1:33 (corresponding to 1:100 of the original antibody) for immunoblotting. Protein assays for each sample were performed using the Pierce Microplate BCA protein assay kit (Thermoscientific).

2.3. Quantitation of TRPA1 immunoreactivity

Digitized images of the blots were acquired using a Epson RX500 scanner and Adobe Photoshop software. Band intensities for TRPA1 immunoreactivity in individual lanes were analysed using NIH ImageJ software, and normalized to loaded protein. P values and statistical significance were determined by the unpaired Student’s t-test using GraphPad Quick-calcs software to compare the values obtained for each post-injury time point with the values obtained for controls. In Fig. 3, results are reported as means ± SEM. *P* < 0.0019 for the 4-day post-injury data, *P* < 0.43 for the 1-day post-injury data, and *P* < 0.20 for the 7-day post-injury data.

3. Results

To analyse the effects of tooth injury on TRPA1 channel expression in the trigeminal ganglia (TG) we made minor modifications to a rodent model for tooth injury that has been used previously. Rats were harvested for analysis at 1, 4, and 7 days after pulp exposure. In addition to dissecting both TG from each animal we also dissected and examined the left and right mandibular molars. Experimental animals showed clear indications of enamel and dentinal damage and pulp exposure of the left mandibular molars (Fig. 1A) when compared to the uninjured molars on the right side (Fig. 1B). Apart from the tooth injury and pulp-exposure, experimental animals did not display any gross signs of inflammation or infection around the site of injury, and also did not display major changes in their food and water intake throughout the course of the experiments. Injury to the left molars could potentially alter TRPA1 expression in the TG on the ipsilateral and contralateral sides. To test this possibility, we first analysed TRPA1 expression in the left (ipsilateral) and right (contralateral) TG from animals 4 days after pulp exposure by Western blot analysis. Since TRPA1 is a membrane protein, we used an extraction procedure for the selective enrichment of membrane proteins, in order to improve sensitivity. Membrane extracts of TG were analysed by immunoblotting with an antibody to rat TRPA1. In animals with injury to their left molars (*n* = 4), TRPA1 protein was detectably increased in the left TG compared to the right TG. The increase in expression of TRPA1 in the left TG in two animals from this group is shown in Fig. 2A. Thus, tooth injury appeared to selectively increase TRPA1 expression in TG innervating the injured teeth, without substantially altering TRPA1 expression in TG innervating the uninjured, contralateral side. To confirm that the antibody was indeed detecting TRPA1 protein, the blot was stripped and reprobed with TRPA1 antibody pre-incubated with a commercially available peptide used to generate the antibody. No bands were detectable with the preadsorbed antibody, confirming that it was selective for TRPA1 (Fig. 2B).

![Fig. 1 – Pulp exposure of mandibular molars. (A) An occlusal view of the mandibular molars shows the extent of tooth injury to the first and second molars after pulp exposure. (B) A corresponding view of the mandibular molars on the uninjured right side is shown for comparison.](image1)

![Fig. 2 – Western blots of membrane extracts of TG innervating injured or uninjured teeth from two animals, R1 and R2, probed with TRPA1 antibodies. (A) TRPA1 immunoreactivity in extracts of left TG (+) innervating the injured mandibular molars was detectably higher than in extracts of right TG (−) innervating the uninjured, contralateral side (*n* = 4). (B) Immunoreactivity was not detectable after preadsorption of TRPA1 antibody with a synthetic peptide used to generate the antibody, indicating that the antibody selectively recognized TRPA1.](image2)
We then compared TRPA1 expression in the left TG from a second group of experimental animals and controls for a period of 1 week following tooth pulp exposure. We selected time points of 1, 4 and 7 days based on the premise that changes in expression of nociceptive modulators like TRPA1 would be most likely to occur in this period. A set of pulp-exposed animals (n = 12) and a control were sacrificed at 1, 4, and 7 days after injury. Membrane extracts of individual left TG from experimental animals and controls harvested at the selected time points were analysed by immunoblotting with the TRPA1 antibody. To assess these changes more accurately we expressed TRPA1 immunoreactivity by normalizing TRPA1 band intensity in each lane to the amount of protein loaded. Data from extracts of individual TG from experimental animals for each time point, and from controls, are shown in Fig. 3. Normalized TRPA1 immunoreactivities obtained for TG samples from the first group of animals represented in Fig. 2 are also included in Fig. 3. We found no statistically significant difference (P < 0.86) between normalized TRPA1 band intensities obtained for right TG extracts from animals with left molar tooth injury (n = 4), and corresponding values obtained for left TG extracts from uninjured controls (n = 3). Therefore, the data obtained for right TG (uninjured side) were included in the data for controls in Fig. 3. Similarly, TRPA1 immunoreactivities from the left TG (injured side) from the first group were included in the post-injury day 4 data in Fig. 3. The average of normalized band intensities from control ganglia was 3.75 ± 0.81. For the ganglia on the ipsilateral side of the tooth injury, average normalized band intensities were 4.9 ± 0.85, 11.42 ± 1.77, 1.92 ± 0.56 at 1, 4, and 7 days, respectively. The average TRPA1 immunoreactivity relative to controls was 1.3-fold, and 3-fold higher at day 1 and day 4 after tooth injury, respectively. At 7 days after tooth injury the average TRPA1 immunoreactivity was 0.5-fold lower relative to controls. Whilst the increase in TRPA1 expression at day 4 after tooth injury was statistically significant compared to controls (P < 0.002), there was no statistically significant variation between the data for TRPA1 expression at 1 day or 7 days after tooth injury and controls. The results indicate that tooth injury leads to a transient, and statistically significant increase in TRPA1 expression in the TG innervating injured teeth.

4. Discussion

Trigeminal neurons are the major source of nociceptive innervations of the dental pulp. Our studies offer evidence that trigeminal neurons undergo changes in expression of TRPA1 channels after dentinal damage and pulp exposure. Expression of TRPA1 was found to increase up to 4 days after injury, decreased by 7 days, and was restricted to the TG on the ipsilateral side. Our results are consistent with results in other models of nerve injury, in which TRPA1 expression and the proportion of cold-responsive Aδ fibres is significantly increased.24,34 The release of pro-algesic and pro-inflammatory agents may further increase TRPA1 activity.24 Uregulation in trigeminal expression of TRPA1 channels after tooth injury could thus contribute to the development of hyperalgesia and cold allodynia, and/or increased sensitization of the pulp to chemical or mechanical irritants in the oral cavity.

There are several possible ways by which a widespread increase in somal expression of TRPA1 channels in TG neurons could occur after tooth injury. One possibility is that pulp exposure increases the number of TRPA1 expressing neurons, and is not restricted to neurons innervating injured teeth. In support of this possibility there is evidence of a widespread increase in the number of TRPV1-expressing trigeminal neurons after tooth injury.35 Exposure to nerve growth factor (NGF) was found to significantly increase the number of neurons coexpressing TRPA1 and TRPV1 in vitro and in vivo.8 NGF levels and NGF receptors are known to be increased in pain-sensing fibres following nerve injury and inflammation.36–38 A widespread increase in TRPA1 channel expression may thus be important for maintaining the appropriate balance in interactions between TRPA1 and TRPV1, since each channel appears to have the capability of regulating the other.27,28 Alternatively, the increase in TRPA1 protein observed in this study and in TRPV1 reported previously39 may originate from increased expression in the neurons that were already expressing the respective channels exclusively, or may be restricted to the neurons innervating the injured teeth. Further studies will be necessary to distinguish between these possibilities. The relative changes after tooth injury in TRPA1 and TRPV1 co-expressed by individual sensory neurons within the ganglion and in sensory endings in the pulp also need to be investigated.

Increased expression of TRPA1 provides a mechanism that might explain the pain and sensitivity of the teeth to cold and environmental irritants after surgery, trauma, or conditions associated with chronic oral pain. A general increase in expression of TRPA1 channels in human trigeminal neurons

![Graph - TRPA1 levels increase transiently during the first week after tooth injury.](image)
may contribute to the hyperalgesia and allodynia after tooth injury. Novel therapeutics capable of selectively targeting TRPA1 are under development and potentially represent a new class of analgesics that may be useful for treating pain and inflammation. Identification of the molecules and cellular mechanisms mediating dental and orofacial pain is essential for developing new therapies aimed at reducing the hyperalgesia associated with injury and inflammation of oral tissues.

Acknowledgements

The authors thank Anthony Elger and Sandra Sawyer for technical assistance, and N. Gautam for comments on the manuscript.

Funding: This work was supported by a grant from the American Association of Endodontists (K. Rowland and M. Gautam) and a Summer Research Fellowship (E. Haas).

Competing interest: None declared.

Ethical approval: Not required.

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