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An Evaluation of the role of the TRPM8 Receptor in mediating cold pain in Rodent Molar Teeth

by

Yvonne F Chiu, DDS

THESIS

Submitted in partial satisfaction of the requirements for the degree of

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in the

GRADUATE DIVISION

of the

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I would like to show my gratitude to my mentors, Dr. Jennifer Gibbs, Dr. Tri Huynh, Dr. Grayson Marshall, and Dr. Arthur Miller, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

Yvonne Chiu

An Evaluation of the role of the TRPM8 Receptor in mediating cold pain in Rodent Molar Teeth.

By Yvonne Chiu, DDS

The transient receptor potential melastatin 8 (TRPM8) receptor is activated by the chemicals menthol and icilin and mediates innocuous and noxious cold. The role of TRPM8 in mediating noxious cold pain in pulpal neurons was assessed in this study. We used immunohistochemistry to evaluate the expression of TRPM8 in mice molars mediating noxious cold. In order to characterize the neuronal population expressing TRPM8, we used a retrograde tracer, Fos to identify pulpal neurons in the trigeminal ganglion. This method combined with multiple label immunohistochemistry identified the neurochemical properties of pulpal afferents that express TRPM8. The results in this study demonstrated that Fos expression in the transition zone neurons in the trigeminal ganglion increases significantly upon noxious cold stimulation in normal pulp of the experimental mouse. This finding suggests that a noxious cold stimulus increases neural activity in relevant brainstem neurons. In the presence of dental pulp injury (i.e., pulpal inflammation), a reduction of Fos expression in the transition zone neurons is observed compared to normal pulp, although not statistically significant. Degeneration of nerve fibers may be the reason why a reduction was seen in our study. The finding that the absence of TRPM8 does not effect cold-evoked Fos expression significantly indicates that TRPM8 is likely not the only receptor responsible for thermosensation in the dental pulp in mice.

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An Evaluation of the role of the TRPM8 Receptor in mediating cold pain in Rodent Molar Teeth

By Yvonne Chiu, DDS

Introduction

Human dental pulp, also referred to as the pulpodentin complex, is one of the most densely innervated tissues in the body. The sensory neurons in the dental pulp respond to temperature as well as to mechanical fluid movement through dentinal tubules, as occurs in conditions of dentinal hypersensitivity. (1-4) Furthermore, pulpal neurons are activated by various inflammatory mediators including both endogenous substances such as bradykinin and nerve growth factor (NGF), as well as non-endogenous activators of nociceptors such as capsaicin. (5-12)

Nociceptors can be broadly divided into two classes. (13-16) First, the A-delta (myelinated) fibers are located in the periphery of the pulp, have a relatively low stimulation threshold and produce sharp, pricking pain. These fibers represent 25-50% of those innervating the dental pulp, dentin, predentin, and odontoblast layers near the pulp horn. (17) In contrast, C (unmyelinated) fibers are located deeper within the pulp. They have higher stimulation threshold that are usually activated with tissue injury, and characterized by dull aching. (18) A-delta and C fiber afferents also contain subpopulations that respond to cooling to innocuous cool temperatures of 15-30C°, to noxious cold at 15C° and below, or noxious heat at 43C° or above. (19) The ability of a human to sense temperature is largely due to several members of a class of receptors termed transient receptor potential (TRP) ion

channels. TRP channels are activated by temperature as well as by certain chemicals such as capsaicin, menthol, mustard oil, camphor, and eugenol. When these chemicals are administered to humans, they elicit burning or cooling sensations. It is these receptors, located in the dorsal root ganglion (DRG) neurons and trigeminal ganglion neurons that serve as warning signals to prevent injuries. For reviews see (20-23). Furthermore, inflammatory mediators such as bradykinin, prostaglandin E2, and NGF modulate these receptors. (24-28) Recently, TRP channels have been implicated in mechanotransduction (29) as well. In this regard, the properties of TRP channels remarkably mirror those of pulpal sensory neurons.

There are many different TRP receptors within the family. The one of particular interest to this project is TRP melastatin 8 (TRPM8). TRPM8 is a ligand gated, non-selective cation channel. *In vitro* studies using cloning and other molecular techniques determined the tissue distribution of TRPM8 to be limited to about 5 to 10% of adult DRG neurons and trigeminal ganglia, particularly small-diameter C-fiber neurons. In addition, TRPM8 was shown to respond to decrease in temperature and the activation range to be between 19C° and 25C° in a voltage clamp study. (30) Finally, menthol was shown to mimic the effect of lowering the temperature on TRPM8 expressing cells. (30-32) Icilin and eucalyptol are also agonists of TRPM8. (30, 33-36) Through these studies, TRPM8 is believed to play a role in cold thermosensation. Analyses of TRPM8-null (TRPM8-/-) mice suggest that it is the principle cold sensor *in vivo*. Behavioral studies using the thermal preference chamber and jumping (escape) behaviors demonstrated that TRPM8-/-

mice showed reduced avoidance to cold temperatures as well as to icilin application. (37) The results are consistent with those of *in vitro* studies.

It is important to remember that TRPM8 is not found to be co-expressed with known nociceptive markers such as calcitonin gene-related peptide (CGRP) and isolectin B4 (IB4). (33) It is, however, found to be co-expressed with TRPV1, an important receptor in mediating inflammatory pain, especially heat hyperalgesia. In addition to innocuous cool, TRPM8 is also involved in transducing noxious cold sensation, but there may be other receptors involved. However, to date the TRPM8 receptor appears to be the primary molecular mediator of cold sensation (For review, see (38).

A sensory stimulus, whether it is thermal, chemical, or mechanical, to the pulp is almost always perceived as pain. The hydrodynamic theory of dentin sensitivity proposes that pain-producing stimuli cause a change in dentin fluid flow that activates A-delta fibers through a mechanoreceptor response, to cause pain. (2, 42-47) However, the recent discovery of TRPs may be an additional mechanism by which thermal stimuli activate nerve endings. (48-51) One can simply determine if particular TRP channels are expressed on myelinated (A-delta fibers) or unmyelinated (C-fibers) pulpal neurons to indicate which type of sensory modality they likely convey.

This study is based on the hypothesis that TRP channels contribute to thermosensation in the dental pulp and play an important role in mediating painful clinical conditions such as pulpitis. With TRPM8 as the receptor of interest, we examined its expression in pulpal neurons of transgenic mice and determined whether it was necessary for noxious cold stimulation of the pulp.

Generation of mice lacking TRPM8 receptors provided us a way to study their roles in thermosensation *in vivo*. The TRPM8 null mice used in this study were engineered using the bacterial artificial chromosome (BAC)-based targeting approach. (52) First, 27 amino acid residues in the start codon of TRPM8 gene were deleted. Farnesylated enhanced green fluorescent protein (EGFPf) was then knocked-in upstream to the start codon of TRPM8. This GFP construct allows visualization of TRPM8 expressing neurons in histological sections, without depending on an antibody to the receptor. This is very advantageous as the antibodies to this receptor are notoriously non-specific. The GFP can be visualized under standard fluorescent microscopy or the signal can be enhanced by using standard immunohistochemistry with an antibody to GFP that is highly specific. In addition to expressing a construct that allows visualization of TRPM8, this targeting construct also prevents transcription of the entire TRPM8 gene and creates a TRPM8 null (-,-) mouse. The construct is cloned into the BAC targeting vector and transfected into embryonic stem cells. Germline transmission of the mutated allele was verified by PCR in F2 mice (Figure 1 adapted from (39, 40). Previous studies have employed the use of such TRPM8-null mice and successfully demonstrated

reduction in behavioral responses to cold stimulation. (39, 40) The presence of TRPM8 was first demonstrated in dental pulp using transgenic GFP mouse model. TRPM8 expression was found on neurons that innervate dentin, most likely A-delta fibers. In humans, TRPM8 expression was also demonstrated in dental pulp. However, its expression was reduced in the presence of irreversible pulpitis, leading the authors to conclude that TRPM8 is most likely not involved in cold-mediated noxious cold pain mechanism. (48) These mice were used in the study and were kindly donated by Dr. Patapoutian.

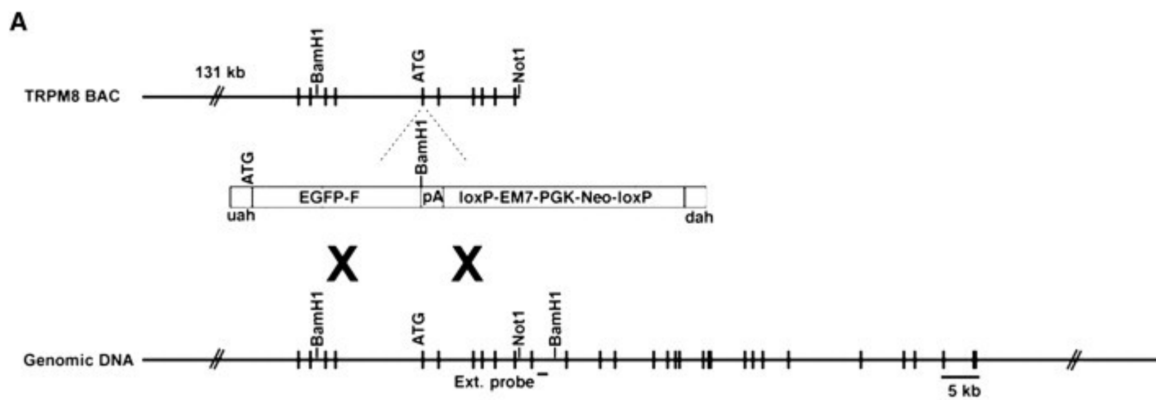


Figure 1. Generation of TRPM8 null mice (adapted from(39, 40).

The gene c-*fos* and its protein Fos were employed as an activity marker in the spinal and brainstem neurons in response to noxious chemical, thermal, and mechanical stimulations. (53-56) Transcription of c-*fos* takes place within minutes following stimulation and its protein expression peaks at about 2 hours. Typically, the gene expression is measured using *in situ* hybridization, whereas the protein expression can be evaluated using immunocytochemical techniques. By using c-*fos,* we can

easily visualize the precise anatomical location of neuronal populations that respond to noxious stimulation. We can quantify the expression simply by counting the number of neurons labeled in anatomical areas of the CNS known to be involved in processing nociceptive trigeminal signals. (57, 58) While using c-*fos* expression has various advantages for studying the neural basis of nociception, it is also important to recognize the limitations. Induction of c-*fos* expression to the levels that can be reliably measured requires strong and prolonged stimulations. In addition, not all neurons express the gene when activated. Therefore, it is critical to recognize that the absence of c-*fos* expression cannot be taken to identify the absence of neural activity. One must be cautious when drawing comparisons between different structures to avoid false negatives. Furthermore, there is also an attendant risk of false positives. The problem arises because it is usually very difficult to determine what exact events were directly responsible for provoking expression of the gene. Perhaps, this is the most important drawback. (57)

In our studies, we evaluated expression of Fos in the trigeminal nucleus, including subdivisions of caudalis, transition zone, and the interpolaris. These areas were chosen because previous studies have shown that Fos is expressed in neurons in the spinal trigeminal nucleus in response to noxious stimulation to the face. (59)

The purpose of this study was threefold: 1) to determine whether noxious cold stimulation to the dental pulp increases Fos expression in the brainstem in wild type mice; 2) to determine if pulpal inflammation alters Fos expression in the brianstem

upon noxious cold stimulation; and 3) to determine whether TRPM8 mediates noxious cold pain in pulpal nociceptors using TRPM8 null mice.

Materials and Methods

Aim 1: to determine whether noxious cold stimulation to the dental pulp increases Fos expression in the brainstem in wild type mice

Animals: Ten adult (>6 weeks old) wild type mice strain C57-BL6 (around 19-21g) of both genders from Charles River Laboratories International, Inc. were used in the experiment.

Cold stimulation to dental pulp: Deep anesthesia was achieved by inhalation of isoflurane and intraperitoneal injection of 2.5% Avertin using a 27-gauge needle. Studies have shown that the uses of the above anesthetic agents on mice are safe and do not affect brainstem neurons firing when compare to ketamine, another commonly used anesthetic. (60-63) An EndoIce saturated cotton pellet was applied directly onto the animal's maxillary left first molar twice every two and a half minutes, repeated 12 times over thirty minutes total. Meanwhile, its contralateral right first molar received no stimulation, serving as control. An operating microscope was used to visually confirm application of the cold stimulus to the maxillary molar. Animals were re-anesthetized as needed to ensure that they remained unconscious until perfusion.

Tissue preparation: Two hours after the initial stimulation, anesthetized mice were transcardially perfused with 20mL of 0.1M PBS followed by 20mL of 4% paraformaldehyde. Following perfusion, the brainstem was dissected and removed, post-fixed in 4% paraformaldehyde at 4C° for up to 4 hrs. The tissues were then cytoprotected in 30% sucrose at 4C° for storage. They were frozen in optimal cutting temperature medium (OCT), sectioned with a cryostat at 40 μm.

Immunohistochemistry: Sections of the brainstem including the trigeminal nucleus from the caudal level of the nucleus caudalis to the rostral level of the interpolaris were collected. Every third section was selected for immunohistochemistry and cell counting. Free floating sections were pre-incubated with 5% NGST blocking solution for 1 hour, then incubated overnight at room temperature with primary antibodies diluted in blocking solution. The tissues were then washed three times in 1% NGST buffer solution for 10 minutes each followed by 0.3% H_2O_2 in 5% NGST for 30 minutes to reduce endogenous tissue peroxidase levels. Tissue sections were then incubated with secondary antibodies made from biotinylated goat anti-rabbit and 1% NGST buffer for one hour. Tissues were washed three times with 0.1M PBS for 10 minutes each. The avidin-biotin-peroxidase method using a commercially available "ABC" kit was then used. Tissues were reacted with DAB with nickel enhancement to elicit the chromogen reaction. Tissues were then mounted onto gelatin-coated slides and dried. Finally, tissues were dehydrated with different concentrations of alcohol and cleared with xylenes and coverslipped.

Cell counting: Slides were observed under microscope at magnification 10X. The number of Fos immunoreactive cells were counted by an independent examiner without prior knowledge of the side of the brain being evaulated. The ipsilateral (stimulated) and contralateral (unstimulated) sides of the trigeminal nucleus were compared. The level of the trigeminal nucleus (caudalis, transition zone, or interpolaris) was also noted, as well as whether the cell appeared in the grey matter or white matter. Data analysis was then performed.

Data analysis: The average number of Fos positive cells per section per mouse between ipsilateral and contralateral of trigeminal nucleus at each individual level was compared by an unpaired, two-tailed Student's t-test ($p<0.05$).

Aim 2: to determine if pulpal inflammation alters Fos expression in the brainstem upon noxious cold stimulation

Animals: Adult (>6 weeks old) wild type mice strain C57-BL6 (around 19-21g) of both genders from Charles River Laboratories International, Inc. were used in the experiment.

Pulpal exposures were created on maxillary first molars bilaterally using a high-speed electric drill with a 1/8 round bur, and left unsealed. Cold stimulation was done on the maxillary first molar on the dental pulp injury (DPI) side after 9 days of

exposure, leaving the other side (naïve) as control. Tissue samples were processed the same way as described above.

Data analysis: Outcome for each animal was the number of Fos positive cells in the ipsilateral trigeminal nucleus minus the number of Fos positive cells in the contralateral. Unpaired, two-tailed Student's t test was used for statistical analysis ($p<0.05$).

Aim 3: to determine whether TRPM8 mediates noxious cold pain in pulpal nociceptors using TRPM8 null mice

Animals: Four adult (>6 weeks old) TRPM8 null mice were used in the experiment. Methods were described as in Aim 1. Breeding and genotyping of offspring from the pair of mice originally donated by Dr. Patapoutian's lab was performed.

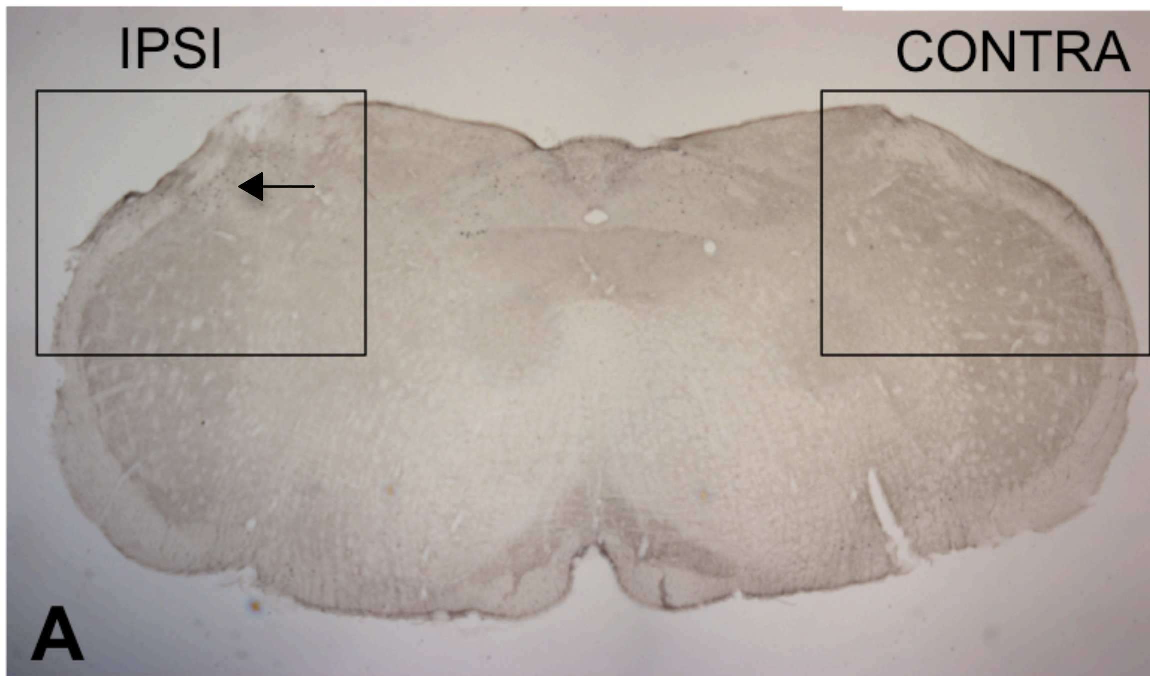
Data analysis: The average number of Fos positive cells per section per mouse between ipsilateral and contralateral trigeminal nuclei at each individual level was compared by two way ANOVA ($p<0.05$).

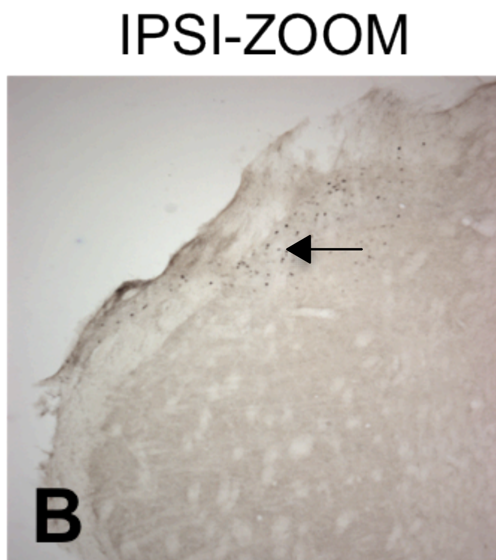
Results

Effect of cold stimulation on Fos expression in wild type mice

No statistical significance was found in Fos expression on the ipsilateral side and contralateral side in nucleus caudalis ($p>0.05$). However, a significant increase in Fos expression was seen on the ipsilateral side (average 4.56 cells/section/mouse)

versus the contralateral side (average 3.42 cells/section/mouse) in the nucleus interpolaris ($p<0.05$). In the transition zone, which is located between nucleus caudalis and nucleus interpolaris, a significant increase in Fos expression was also seen on the ipsilateral side (average 50 cells/section/mouse) than on the contralateral side (average 18 cells/section/mouse) ($p<0.05$; Figures 2-5).





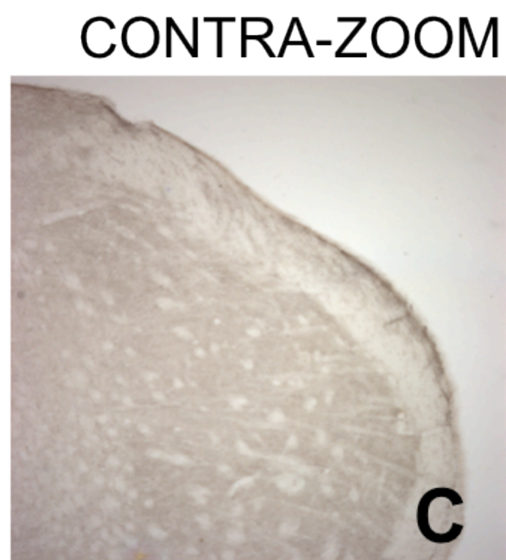


Figure 2. A) Histological section of brainstem at the level of transition zone showing Fos expression (see arrow). B) Magnification of the ipsilateral (experimental) side showing robust Fos expression, see arrow. C) Magnification of the contralateral (control) side with less Fos cells.

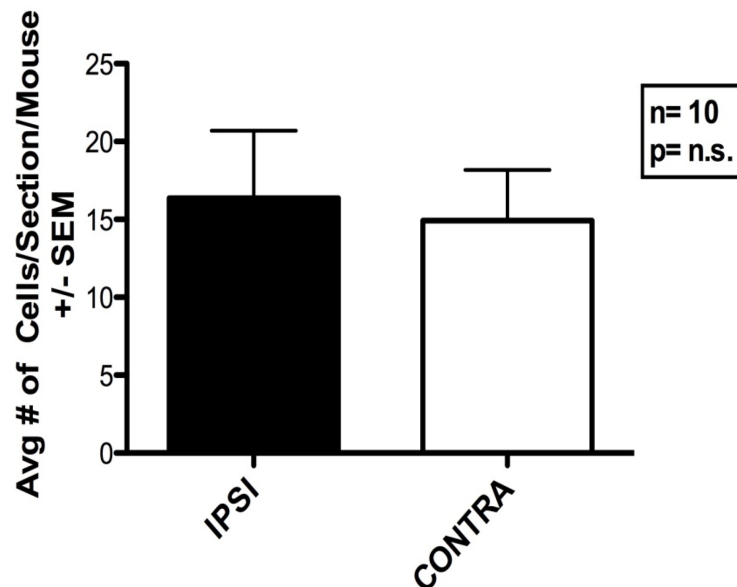


Figure 3. **Effect of cold stimulation on Fos expression in nucleus caudalis in wild-type mice.** N= 10. No statistical significance was found in Fos expression on the ipsilateral side (black bar) and contralateral side (white bar) ($p>0.05$).

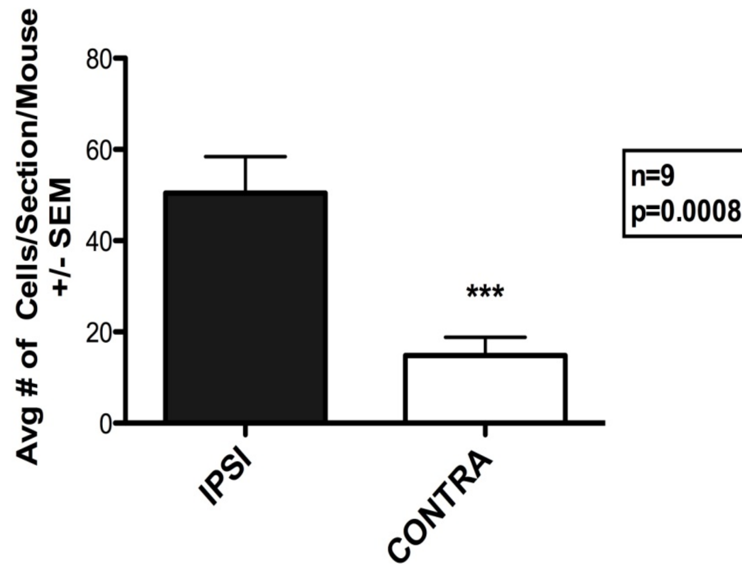


Figure 4. **Effect of cold stimulation on Fos expression in Caudalis/Interpolaris transition zone in wild type mice.** N=9. A significant increase in Fos expression is seen on the ipsilateral side (black bar) than on the contralateral side (white bar) ($p<0.05$).

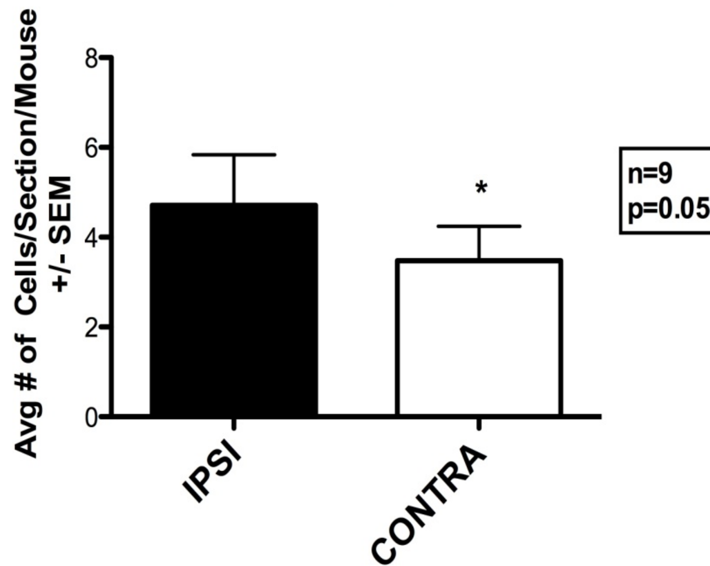


Figure 5. **Effect of cold stimulation on Fos expression in nucleus interpolaris in wild-type mice.** N=9. A Significant increase in Fos expression is seen on the ipsilateral side (black bar) versus the contralateral side (white bar) ($p<0.05$).

Effect of dental pulp injury (DPI) on cold evoked Fos expression in wild type mice

Dental pulp injury showed a reduction of Fos expression in the transition zone neurons compared to normal pulp, although not statistically significant ($p>0.05$; Figure 6).

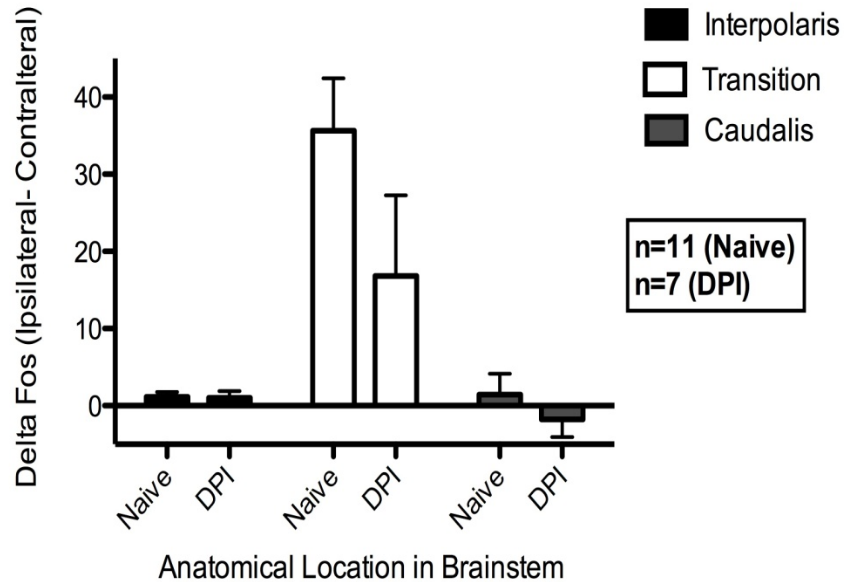


Figure 6. **Effect of dental pulp injury (DPI) on cold evoked Fos expression.** N=11 (Naïve) and N=7 (DPI). No difference in delta Fos (ipsilateral minus contralateral) is observed between naïve and DPI for both nucleus interpolaris (black bars) and nucleus caudalis (grey bars). A reduction in Fos expression is seen on the DPI side in the transition zone (white bars), albeit not statistically significant.

Effect of cold stimulation on Fos expression in TRPM8 null mice

Repeated cold stimulation of the mouse molar showed similar Fos expression pattern as wild type mice. Increase in Fos expression is seen on the ipsilateral side. However, the difference is not statistically significant ($p>0.05$; Figure 7).

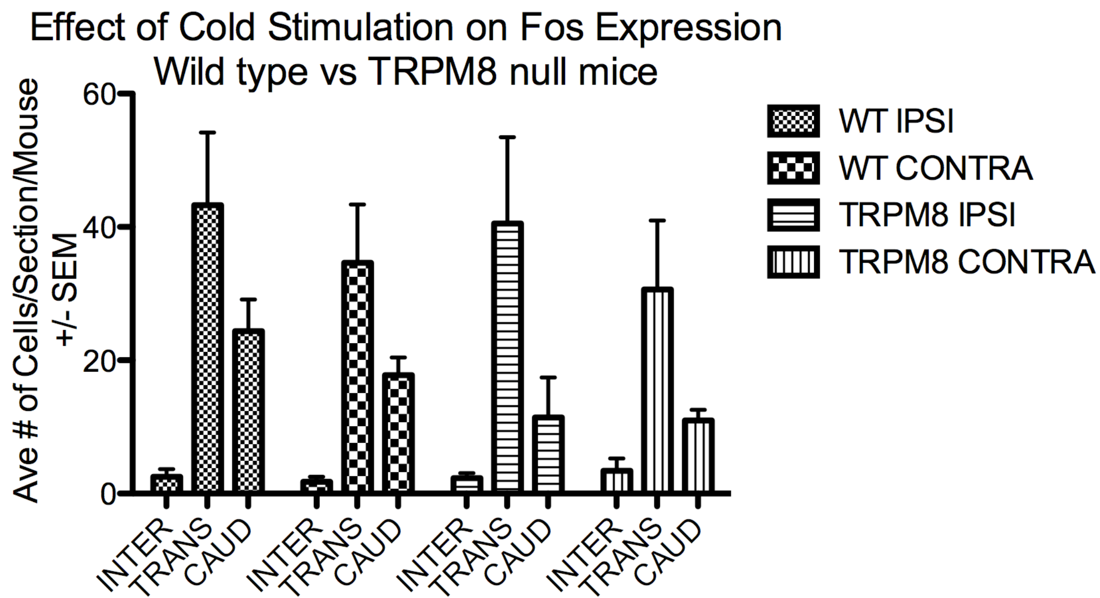


Figure 7. **Effect of cold stimulation on Fos expression between wild type and TRPM8 null mice.** N= 4 (wild type mice) N=4 (TRPM8 null mice). Wild type mice: an increase in Fos expression is seen on the ipsilateral side compared to the contralateral side at all three anatomical locations. TRPM8 null mice: same Fos expression pattern is observed. Statistically, there is no significant difference between wild type mice and TRPM8 null mice in sides or anatomical locations.

Discussion

The results in this study demonstrated that Fos expression in the transition zone neurons increases significantly upon noxious cold stimulation in normal pulp of the experimental mouse. This finding suggests that a noxious cold stimulus increases neural activity in relevant brainstem neurons. The model developed in this study can be used to study pain transduction from the dental pulp. Using Fos expression as an outcome measure was a valid tool in this study. As mentioned previously,

strong and prolonged stimulation is required for induction of c-*fos*. Even though we cannot precisely measure the amount of cold stimulus applied to the mouse's molar, it is clearly demonstrated from the first aim of the study that Fos expression increases significantly in the presence of noxious cold stimulation. Hence, the amount of stimulus applied as well as the time of application was adequate.

In the presence of dental pulp injury (*i.e.,* pulpal inflammation), a reduction of Fos expression in the transition zone neurons is observed compared to normal pulp, although not statistically significant. Nine days post-dental pulp injury was chosen as the time point to perform cold stimulation because chronic inflammation simulates the clinical situation (*i.e.,* irreversible pulpitis). In a study that evaluated the histology of dental pulps in crown-fractured teeth, myelin degeneration surrounding the axon and edema was seen in the early stage post-trauma (17h). In the later stages (4 to 20 days), the tissues showed varying degrees of inflammation, and neuronal degeneration such as intramyelin edema, aberrant myelin synthesis, and axonal swelling. (64) Hence, degeneration of nerve fibers may be the reason why a reduction was seen in our study. If the pulp status were necrotic by nine days, then one would expect cold-evoked Fos expression to be minimal to none as there are no sensory neurons remaining in the pulp. Based on this finding, it is suggested that inflammation does not play a role in thermosensation in this experimental model. Future studies using a shorter time point (less than 9 days) and a longer time point (more than 9 days) post-dental pulp injury will be useful in determining whether acute inflammation and pulpal necrosis alter noxious cold-

evoked Fos expression. Also, histological examination of the pulp taken from different time points should be performed to confirm the pulp status.

Finally, the finding that the absence of TRPM8 does not effect cold-evoked Fos expression significantly indicates that TRPM8 is likely not the only receptor responsible for thermosensation in the dental pulp in mice. The contributions of other cold transduction molecules should be considered, such as potassium and sodium channels. (65, 66) Recent studies by El Karim successfully demonstrated that human dental pulp fibroblasts and odontoblasts express TRP channels, specifically, TRPA1 and TRPM8 at the molecular, protein, and functional levels. Their results indicate possible roles of both fibroblasts and odontoblasts in mediating cold responses in human teeth. (67, 68) In addition, one should consider the species difference and note that the distribution of TRP channels are not the same between human and mouse. (69)

A potential limitation of the study is the number of TRPM8 null mice used. Statistically, a lower sample size weakens the power of the study. By increasing the number of TRPM8 null mice, we can increase the power of the conclusion drawn from the observation. Further research is warranted to extrapolate clinical relevance of the findings from this study.

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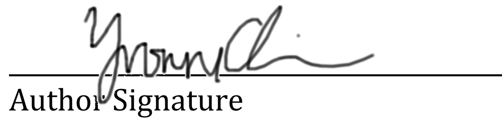

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