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Quantification of Neural Protein in Extirpated Tooth Pulp

Curt A. Warren, DDS¹, LeePeng Mok, MS², Sharon Gordon, DDS, MPH, PhD², Ashraf F. Fouad, DDS, MS¹, and Michael S. Gold, PhD

¹Department of Endodontics, University of Maryland Dental School, Baltimore, MD 21201

²Department of Biomedical Sciences, University of Maryland Dental School, Baltimore, MD 21201

Abstract

Because the pulp tissue extirpated during root canal procedures may serve as a valuable resource with which to assess underlying mechanisms of persistent pain, we sought to determine whether standard western blotting techniques could be employed to quantify neural proteins in pulp extirpated from teeth with irreversible pulpitis. Pulp harvested from healthy intact teeth extracted for orthodontic reasons was used for comparison. The neural marker PGP9.5 was detectable in all samples tested. A membrane enrichment protocol enabled detection of even low abundance, high molecular weight proteins such as the sodium channel α -subunit Na_v1.8. Importantly, it was possible to quantify a ~6-fold increase in the relative density of Na_v1.8 in inflamed pulp compared to control pulp. Our results suggest that it should be possible to use extirpated tooth pulp to validate mechanisms of persistent pain implicated in pre-clinical studies as well as evaluate the therapeutic efficacy of novel antinociceptive interventions.

Keywords

Nociceptor; primary afferent; pain; NaV1.8

Introduction

Pain continues to be a major health problem with tremendous financial, social and psychological costs. Yet, there remain few if any effective therapies devoid of serious side effects that are currently available to treat pain, particularly pain that persists following tissue injury. While our understanding of mechanisms underlying persistent pain has been advanced through the use of a number of animal models, it is necessary to validate observations made in model systems in humans (1). This form of validation is often difficult, particularly in the case of persistent pain syndromes, where access to relevant tissue is limited. Because there is an extensive body of both clinical and pre-clinical data suggesting that persistent pain reflects, at least in part, changes in the peripheral nervous system (2), access to peripheral neural tissue may be particularly important.

According to the American Dental Association, almost 16 million root canal treatment procedures are performed in the United States every year (3). This procedure is generally performed as a treatment of irreversible pulpitis or pulp necrosis, the former being a condition

Corresponding Author: Michael S. Gold, PhD, Department of Medicine, University of Pittsburgh, 3500 Terrace Street Rm E1440 BST, Pittsburgh, PA 15213, Phone (412) 383-5367, Fax: (412) 383-8663, e-mail: goldm@dom.pitt.edu.

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associated with moderate to severe pulpal pain that may be present for months prior to treatment. Given that pulp tissue is heavily innervated (4), this tissue may provide a valuable source of material with which to assess the presence of neural proteins that may contribute to persistent pain associated with this condition. Because changes in the relative density of neural proteins may be critical for the manifestation of persistent pain (2), it is important to be able to quantify protein levels. Therefore, the following study was performed in order to determine whether it is possible to quantify neural proteins in pulp extirpated from teeth with irreversible pulpitis, as well as assess changes in protein density in extirpated tooth pulp relative to the protein density in pulps from control teeth.

Materials and Methods

Subjects and tissue collection

The use of human subjects in this study was performed in accordance to with standards established by the National Institutes of Health for the use of human subjects in research and was approved by the University of Maryland, Baltimore Institution Review Board.

Two groups of subjects were studied. One was a pain group, selected from subjects presenting in pain to the University of Maryland Dental School endodontic clinic. These subjects were considered for inclusion in the pain group if they presented with moderate to severe preoperative pain, and the teeth involved responded with lingering pain to thermal tests. Healthy controls constituted the second group. Subjects for this group were recruited from an oral surgery clinic and the teeth included were intact non-carious teeth that were extracted for orthodontic reasons. The same exclusion criteria were employed for both groups. These included: history of neurologic disease of any kind, presence of ongoing medical treatment involving the long-term use of analgesics, steroids, or any other pain perception altering drugs, sensitivity to lidocaine or preservatives in the local anesthetic solution, and the presence of factors indicating that the use of epinephrine in local anesthetics was contraindicated.

Subjects satisfying eligibility criteria gave consent for participation in the study, and furnished data including age, sex, and race. Data relevant to each patient's medical history was also collected. Pain data was then collected using a visual analog scale (VAS). Subjects were instructed to place a mark on a 10 cm line (anchored on the left with "no pain" and on the right with "worst pain imaginable") corresponding to 1) current pain 2) the maximum pain experienced in association with the problem, and 3) the average pain for the duration of the problem. A "cold test" was used to assess thermal sensitivity. This involved applying a cotton pellet sprayed with Endo Ice® to the affected tooth. Patients were instructed to raise their hand when they detected the stimulus and to lower it when the sensation disappeared. "Lingering" was defined as the presence of pain that persisted (seconds to minutes) beyond the removal of the stimulus. Finally, clinical data was collected, including diagnostic testing data. Endodontic procedures were performed by second-year endodontic residents at the University of Maryland Dental School.

Anesthesia was induced with 2% lidocaine with 1:100,000 epinephrine and the amount administered in carpules was tracked for each patient. Prior to initiating procedures to access the pulp chamber, all patients demonstrated a positive soft tissue response. Labial gingiva was used to assess anesthesia of maxillary teeth and the lower lip was used to assess anesthesia of mandibular teeth. A positive soft tissue response was the total loss of sensation in response to probing. Maxillary and mandibular teeth were studied in equal numbers. Following anesthesia and pulp chamber access in the pain group, pulp tissue was collected in sterile 1.5 ml tubes. Tissue was collected in a number of tubes so that each time a tool containing tissue was removed from the tooth, the tissue could be deposited in a tube and immediately placed in dry ice/ethanol mixture or liquid nitrogen. Once all tissue was collected, tubes were transferred to a -80° C

freezer for storage prior to processing. For the control group, teeth were collected immediately after extraction. A groove was placed with a surgical handpiece (3 mm deep extending across the occlusal surface of the entire crown and in the occlusal groove). Teeth were flash frozen in liquid nitrogen, and then stored in dry ice until they could be split open to collect the pulp tissue. A hammer and chisel were used to split the teeth, the chisel being placed in the prepared groove. Collected pulp tissue was then placed in dry ice and transferred to a -80°C freezer for storage.

Western Blot analysis

Membrane protein enrichment—4 to 5 mg of pulp tissue was crushed with a plastic pestle in 200 µl of homogenization buffer, containing TE (50mM Tris-Cl pH8.0 and 10mM EGTA), protease and phosphatase inhibitors (4ng of aprotinin, leupeptin, pepstatin, E64 and trypsin inhibitor per ml; 2mM of phenylmethylsulfonyl fluoride, sodium orthovanadate and 20mM sodium fluoride(5)). Another 200 µl of homogenization buffer was added. Homogenate is pre-cleared by centrifuging at 500g for 5 min at 4°C, followed by ultra-centrifuging at 100,000 g for 1 hour. Of the supernatant, 300 µl was removed; and the pellet was resuspend in 1 µl of 20% SDS and the remaining supernatant. Protein concentration was determined with the Bio-Rad DC Protein Assay before it was denatured at 37°C for 30 min in 1X Laemmli buffer (Bio-Rad). Proteins were separated by SDS-PAGE with a discontinuous buffer system in 10% polyacrylamide gel and then transferred (Bio-Rad semi-dry transfer system protocol) to a nitrocellulose membrane (Schleicher and Schuell Bioscience). Gel casting and running was performed following standard procedures (6). Nonspecific protein binding was blocked with 5% nonfat dry milk in TBST (10mM Tris-HCl pH 8.0, 150mM sodium chloride and 0.05% Tween 20). Blots were incubated at 4°C overnight in primary antibodies (at the appropriate dilution), then in horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch; 1:10,000). Signals were detected using an enhanced chemiluminescence (ECL plus) detection kit from GE Healthcare with a CCD camera (Fuji Medical Systems).

Total protein—Same as shown above except that RIPA (50mM Tris-HCl pH 7.5, 150mM sodium chloride, 1mM EDTA, 1% Triton X-100, 0.1% SDS) with protease and phosphatase inhibitors and 1% of sodium deoxycholate was used as lysis buffer. Tissue was incubated on ice for 2 hours after lysing with intermittent mixing. Protein was denatured by boiling the lysates in 1X Laemmli buffer for 5 min before loading. Primary antibodies employed included: PGP9.5 (Abcam) for detection of neural protein, Na⁺/K⁺ ATPase α subunit (Sigma-Aldrich, St Louis, MO) as independent marker with which to assess membrane enrichment, the Na⁺ channel α subunit Na_v1.8 (Generous gift of Birgit Priest of Merck Inc.) was used to assess changes in a neural protein thought be associated with inflammatory pain (7). This is a polyclonal antibody raised in rabbit against a human specific sequence of the protein (amino acids 2-19). The antibody was not affinity purified. Characterization of the antibody is described elsewhere (8).

Data analysis

Chemiluminescent signals were analyzed with MultiGauge Software (Fuji Medical Systems). Data was normalized with respect to Na⁺/K⁺ ATPase to control for protein loading and transfer and PGP9.5 to control for changes in innervation density. Statistical comparisons were made with SigmaStat software (V3, SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Data were collected from 6 pulpitis and 9 control subjects. Subjects did not vary significantly in demographic variables. The mean age of the pulpitis group was 38.7 ± 6.6 yrs while that of

the control group was 29.8 ± 5.3 years. The pulpitis group had five females, while the control group had 6. The racial makeup of the pulpitis group was 3 African American, 1 Asian and 2 Caucasian, while that of the control group was 2 African American, 2, Asian and 5 Caucasian.

The median duration of the pain reported by the pulpitis subjects was 36 days (14 days minimum and 365 days maximum) with a average VAS score of 6.5 ± 0.7 (mean $\pm$ SEM) for the duration of the problem, a maximum VAS score of 8.1 ± 0.6 and a VAS score at the time of the procedure of 6.5 ± 0.7 . As expected, none of the control subjects reported a VAS above 0 at the time of the procedure. The amount of local anesthetic necessary to produce a positive lip sign was significantly greater ($p < 0.05$, Rank sum test) in pulpitis subjects (median 2.5 carpules with 2 and 3 carpules as the 25th and 75th percentile) than that used in control subjects (median 1 carpules with 1 and 1.25 as the 25th and 75th percentile).

No less than 7 mg of total tissue was obtained from each tooth, permitting several blots to be obtained with tissue from one subject. The non-specific neuronal marker PGP9.5 (9) was used to quantify the amount of neural protein recovered from pulpal tissue. A representative blot of pulp tissue from 3 control and 3 inflamed subjects is shown in Figure 1A. β -actin was used as a loading control with which to normalize data. Pooled data indicate that there was no difference between control and pulpitis subjects with respect to the relative amount of neural protein in pulp tissue (Figure 1B).

Because many neural proteins that appear to contribute to ongoing pain are membrane bound (2), we sought to determine whether it was possible to enrich the membrane fraction of the protein extracted from pulp tissue. Fractionation protocols are generally employed for this purpose as a means of completely isolating membrane and cytosolic fractions. However, these protocols generally require a large amount of starting material and fractionation is often followed by a concentration step prior to gel electrophoresis. The limited amount of starting material proved to be the primary constrain in the present study, particularly for the extirpated pulp, where the amount of soluble protein recovered was considerably less than the weight of the starting material. The enrichment protocol ultimately employed was a hybrid of a more traditional fractionation protocol (10) and a less traditional enrichment protocol (11). Because the protocol employed would not result in the complete separation of membrane and cytosolic fractions, we assessed the efficacy of the protocol by probing blots for the membrane bound α subunit of the Na^+/K^+ -ATPase. Because the membrane fraction was not purified, it was still possible to use β -actin as a loading control. A typical blot of data from the control subjects in which pulpal tissue was divided prior to processing, is shown in Figure 2. Half the tissue was subjected to the enrichment protocol and the second half was processed for total protein. While there was considerable variability in the amount of Na^+/K^+ -ATPase recovered from pulpal tissue, subjecting the tissue to the enrichment protocol resulted in a statistically significant ($p < 0.05$, paired t test) increase in the relative amount of membrane bound protein.

Previous immunohistochemical data suggests that there is an increase in the tetrodotoxin (TTX) resistant voltage-gated Na^+ channel α -subunit, $\text{Na}_v1.8$ in painful human tooth pulp (12). We therefore sought to validate the use of Western blot analysis for the quantification of membrane bound proteins thought to contribute to dental pain by quantifying $\text{Na}_v1.8$ immunoreactivity in pulp from control subjects and subjects with pulpitis. It was not possible to detect the presence of $\text{Na}_v1.8$ in blots of total protein (data not shown), therefore, tissue from both groups was subjected to the enrichment protocol and Na^+/K^+ -ATPase was used as a loading control. Na^+/K^+ -ATPase was used as a loading control in order to normalize $\text{Na}_v1.8$ levels against a membrane bound protein rather than a cytosolic protein such as β -actin or PGP9.5. While $\text{Na}_v1.8$ was barely detectable in control pulp (Figure 3A), it was clearly present in inflamed pulp. As illustrated by pooled data, the nearly 6-fold increase in relative $\text{Na}_v1.8$ levels was statistically significant (Figure 3B, $p = 0.03$, Student's t test). Of note, the relative increase in

Nav1.8 expression is likely an overestimate resulting from the low level of Na⁺/K⁺ ATPase detected. Nevertheless, as can be seen in Figure 3A, the density of bands corresponding to Nav1.8 were still greater in inflamed than control pulp, despite the fact that the same amount of total protein was loaded into each lane prior to SDS PAGE.

Discussion

The purpose of this study was to determine whether it is possible to employ standard western blot techniques to quantify neural proteins in extirpated tooth pulp, and if so, whether it is possible to use this approach to quantify changes in proteins that may contribute to persistent dental pain. We demonstrated that it is possible to detect neural protein in extirpated tooth pulp, from patients who had suffered from dental pain for as long as a year. With a membrane enrichment protocol it is possible to enrich the fraction of membrane bound protein, enabling detection of even low abundance high molecular weight proteins. Finally, using this approach we were able to demonstrate that painful pulpal inflammation is associated with close to a 6-fold increase in the relative abundance of Nav1.8.

There are a number of advantages to the use of extirpated inflamed pulp to quantify changes in proteins in pulp tissue. Root canal therapy is an extremely common intervention performed in an out-patient setting under local anesthesia, all enabling relatively easy access to tissue. The procedure is generally quick in the hands of a skilled clinician, facilitating the rapid processing of tissue and therefore minimizing protein degradation. A relatively large amount of tissue may be harvested, facilitating analysis of multiple proteins. It is possible to perform psychophysical studies to explore aspects of the pain, such as quality, intensity, provoking stimuli, etc, in order to facilitate correlating changes in specific proteins with specific aspects of the perception of pain. Given the duration of the pain problem for many subjects undergoing root canal therapy, access to this tissue may provide unique insight into mechanisms of persistent pain. And while it may be difficult to get patients to agree to a delay in their treatment, it may be possible to validate the efficacy of preceding interventions by assessing changes in specific pulp proteins.

Western blot analysis of protein in extirpated tooth pulp is not, however, without its limitations. While vitality testing may suggest the presence of vital pulp prior to initiating the procedure, and analyzing protein levels relative to a marker such as PGP9.5 may mitigate the impact of this limitation, there may still be extensive necrosis and therefore loss of neural tissue. Such loss may contribute to the variability observed in levels of Na⁺/K⁺ ATPase. Some procedures are more difficult than others so that even in the hands of a skilled clinician, there may be significant degradation of pulp protein. A number of potentially relevant proteins, such as opioid receptors, are present on several different cell types, so it may be necessary to combine western blot and immunohistochemical analysis in order to determine the source of the proteins detected with Western blot.

We chose to focus on Nav 1.8 in the present study for 2 main reasons. First, there is considerable evidence from both pre-clinical and clinical studies suggesting that this Na⁺ channel subunit may contribute to inflammatory hyperalgesia and ongoing pain associated with inflammation (for a summary of this evidence see (13)), including that associated with pulpal inflammation (12). Second, as mentioned above, there is immunohistochemical evidence suggesting that in painful teeth there is an increase in Nav1.8-like immunoreactivity in pulpal afferents (12). That Nav1.8 expression was restricted to pulpal afferents in this previous study and was detected in the present study confirms the utility of the western blot approach in the detection of large and relatively low abundance proteins. The increase in relative levels of Nav1.8 observed in the present study is consistent with results of this previous immunohistochemical study. Although it is important to note that differences between control and inflamed pulp with respect

to the relative density of Nav1.8 may reflect, at least in part, differences in the procedures employed for the collection of inflamed and control tissue. Given that the procedures used for pulp collection from extracted teeth (i.e., rapid freezing and extraction of frozen pulp) should be more conducive to maintaining protein stability, we suggest that the impact of the differences in tissue collection in the present study are minimal. The magnitude of the increase in Nav1.8 in pulp from subjects with moderate to severe levels of ongoing pain adds more weight to the suggestion that blocking this channel may be an effective and selective mechanism for pain relief (14). It is also worth noting that the increase in Nav1.8, a Na⁺ channel isoform may render the tissue relatively insensitive to local anesthetics (15) and may contribute to decrease in local anesthetic efficacy observed in present study as well in reported by others (16).

In summary, we have provided compelling evidence to support the suggestion that standard western blot techniques may be used to quantify neural proteins in extirpated inflamed and normal tooth pulp. Using this approach, we have also provided more evidence in support of the suggestion that Nav1.8 contributes to inflammatory pain. Importantly, it should be possible to use extirpated tooth pulp to validate mechanisms of persistent pain implicated in pre-clinical studies as well as evaluate the therapeutic efficacy of novel antinociceptive interventions.

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References

1. Gold, MS.; Chessell, I.; Devor, M.; Dray, A.; Gereau, RWt; Kane, S., et al. Peripheral nervous system targets: Rapporteur report. In: Campbell, JN.; Basbaum, AI.; Dray, A.; Dubner, R.; Dworkin, RH.; Sang, CN., editors. *Emerging Strategies for the Treatment of Neuropathic Pain*. Seattle: IASP Press; 2006. p. 3-36.
2. Caterina, MJ.; Gold, MS.; Meyer, RA. Molecular biology of nociceptors. In: Hunt, S.; Koltzenburg, M., editors. *The neurobiology of pain*. Oxford: Oxford University Press; 2005. p. 1-33.
3. American Dental Association. The 1999 survey of dental services rendered. 16. American Dental Association; 2002.
4. Byers MR. Dental sensory receptors. *Int Rev Neurobiol* 1984;25:39–94. [PubMed: 6206015]
5. Wesley CS. Notch and wingless regulate expression of cuticle patterning genes. *Mol Cell Biol* 1999;19(8):5743–5758. [PubMed: 10409762]
6. Sambrook, J.; Russell, DW. *Molecular Cloning: A Laboratory Manual*. 3. Cold Spring Harbor, NY: Cold Spring Harbor Press; 2001.
7. Gold MS. Sodium channels and pain therapy. *Cur Op Anaesthesiol* 2000;13(5):565–572.
8. Liu CJ, Priest BT, Bugianesi RM, Dulski PM, Felix JP, Dick IE, et al. A high-capacity membrane potential FRET-based assay for Nav1.8 channels. *Assay Drug Dev Technol* 2006;4(1):37–48. [PubMed: 16506887]
9. Oaklander AL. The density of remaining nerve endings in human skin with and without postherpetic neuralgia after shingles. *Pain* 2001;92(12):139–145. [PubMed: 11323135]
10. Grieco TM, Malhotra JD, Chen C, Isom LL, Raman IM. Open-channel block by the cytoplasmic tail of sodium channel beta4 as a mechanism for resurgent sodium current. *Neuron* 2005;45(2):233–244. [PubMed: 15664175]
11. Gupta A, Edwards JC, Hruska KA. Cellular distribution and regulation of NHE-1 isoform of the Na⁺/H exchanger in the avian osteoclast. *Bone* 1996;18(2):87–95. [PubMed: 8833201]
12. Renton T, Yiangou Y, Plumpton C, Tate S, Bountra C, Anand P. Sodium channel Nav1.8 immunoreactivity in painful human dental pulp. *BMC Oral Health* 2005;5(1):5. [PubMed: 16001984]
13. Gold MS, Flake NM. Inflammation-mediated hyperexcitability of sensory neurons. *Neurosignals* 2005;14(4):147–157. [PubMed: 16215297]

14. Jarvis MF, Honore P, Shieh CC, Chapman M, Joshi S, Zhang XF, et al. A-803467, a potent and selective Nav1.8 sodium channel blocker, attenuates neuropathic and inflammatory pain in the rat. *Proc Natl Acad Sci U S A* 2007;104(20):8520–8525. [PubMed: 17483457]
15. Brau ME, Elliott JR. Local anaesthetic effects on tetrodotoxin-resistant Na⁺ currents in rat dorsal root ganglion neurones. *Eur J Anaesthesiol* 1998;15(1):80–88. [PubMed: 9522146]
16. Hargreaves KM, Keiser KF. Local anesthetic failure in endodontics: mechanisms and management. *Endodontic Topics* 2002;1(1):26–39.

Figure 1.

The neural marker PGP9.5 was detectable in pulp tissue obtained from control subjects (Control) and from subjects undergoing root canal therapy (Inflamed) (A). Each lane was loaded with tissue obtained from a different subject. The blot was first probed with an antibody against PGP9.5, stripped and then re-probed with an antibody against β -actin. The expected molecular weight for PGP9.5 is indicated by the arrow. The intensity of the PGP9.5 band was normalized to that of β -actin and data for control and inflamed pulps were pooled and plotted as a mean $\pm$ SEM (B). There was no statistically significant difference in the relative amount of PGP9.5 pulps from these two groups.

Figure 2.

The membrane bound α -subunit of the Na^+/K^+ ATPase (ATPase) was used to monitor the effectiveness of a protocol for the enrichment of membrane bound proteins. **A.** Pulp tissue from 3 control subjects was divided and subjected to either the membrane enrichment protocol (Enriched) or processed for total protein (Total). Thus, protein loaded in lanes 1 and 4, 2 and 5, and 3 and 6 were from the same subjects. The blot was first probed with an antibody against ATPase, stripped and then re-probed with an antibody against β -actin. **B.** Pooled data suggests the enrichment protocol resulted in a ~3-fold increase in the relative amount of ATPase detected.

Figure 3.

Pulpal inflammation is associated with a dramatic increase in Na_v1.8. **A.** pulp tissue obtained from control subjects (Control) and from subjects undergoing root canal therapy (Inflamed) was subject to the membrane enrichment protocol. Blots were first probed with an antibody against Na_v1.8 (expected molecular weight is indicated by the arrow), stripped and re-probed for the α -subunit of the Na⁺/K⁺-ATPase (ATPase). **B.** Pooled data from control and inflamed pulp indicates that there is a ~6-fold increase in the relative abundance of Na_v1.8 in inflamed pulp ($p = 0.03$).