Sodium channel Na\textsubscript{v}1.8 immunoreactivity in painful human dental pulp

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Abstract

Background

The tetrodotoxin-resistant voltage-gated sodium channel Na\textsubscript{v}1.8 (SNS1/PN3) is expressed by nociceptors and may play a role in pain states.

Methods

Using specific antibodies for immunohistochemistry, we studied Na\textsubscript{v}1.8 - immunoreactivity in human dental pulp and lingual nerve in relation to the neuronal marker neurofilament. Human tooth pulp was extracted from teeth harvested from a total of twenty-two patients (fourteen without dental pain, eight patients with dental pain).

Results

Fibres immunoreactive for Na\textsubscript{v}1.8, were significantly increased on image analysis in the painful group: median (range) Na\textsubscript{v}1.8 to Neurofilament % area ratio, non-painful 0.059 (0.006- 0.24), painful 0.265 (0.13-0.5), $P = 0.0019$.

Conclusion

Na\textsubscript{v}1.8 sodium channels may thus represent a therapeutic target in trigeminal nerve pain states.

Background

Neurogenic pain involves several molecular mechanisms including ion channels, particularly the voltage-gated sodium channels [1, 2]. These are expressed by nociceptor fibres, and are related to the development of ectopic neural activity within the injured nerve. Voltage-gated sodium channels play key roles in the pathophysiology of pain and are distinguished according to their sensitivity to the neurotoxin tetrodotoxin (TTX) as fast-activating TTX-sensitive (TTX-S) channels, or slow-inactivating TTX-resistant channels (TTX-R). The distribution and pathophysiology of these channels, particularly Na\textsubscript{v}1.8, have been the focus of research in pain mechanisms [1]. Recently, antisense treatment blocking this channel reduced neuropathic pain [3]. We have previously described the temporal and spatial distribution of Na\textsubscript{v}1.8 in human sensory neurones [4]; the channels were decreased acutely in sensory cell bodies after spinal cord root avulsion but accumulated in fibres proximal to the site of injury in brachial plexus trunks, and in neuromas. Furthermore, nerve terminals in distal limb neuromas and skin from patients with chronic local hyperalgesia and allodynia all demonstrated marked increases of Na\textsubscript{v}1.8, suggesting that the former may be related to the persistent hypersensitive state.

Pain is the most common symptom of diseased tooth pulp, often a result of coronal caries of the tooth. Tooth pulp afferents fire to a range of stimuli, including temperature, chemical and localized mechanical pressure. Excitation of tooth pulp afferents generates a predominantly nociceptive response [5]. The sensory innervation of tooth pulp has been described previously [6]. Afferent A\textdelta and C-
fibres enter the tooth pulp via the root canal; many terminate in the inner parts of the dentinal tubules, close to the odontoblast processes in the coronal region, forming a subodontoblast plexus [7]. Strong correlations have been reported between the afferent discharge frequency of human pulp nociceptors and pain levels [8]. Many suggestions have been made for the origin of pulpal pain e.g. pulp inflammation involving several mediators located within the pulp (cholinergic and adrenergic neurotransmitters, prostaglandins and cyclic AMP). However, thus far, no correlation has been established between pain characteristics and histology of the pulp [9, 10].

This study aimed to assess if pulpal pain associated with caries, and neuropathic pain subsequent to lingual nerve injury, was associated with any change of \( \text{Na}_{\text{v}}1.8 \)-immunoreactivity within nerve fibres.

**Methods**

Patients scheduled for dental extraction at Guy’s Dental Institute, London, were included in the study, subsequent to providing consent in accordance with the local research ethics committee.

22 permanent molar teeth about to be extracted were tested, 1 hour prior to extraction, for vitality using an electric pulp tester (analytic technology constant current at the mid-buccal surface of the tooth) and with ethyl chloride to confirm the neural vitality of the dental pulp. A pain history was also collected (existing pain and duration). The patients were divided into two groups, those with existing pain and those with no history of pain. The patients were divided into two groups, those with existing pain from the tooth (n=8 patients age range: 40.3 ± 4.0 years) and those with no history of or existing pain (n=14 patient age range: 37.3 ± 14.6 years). The gender distribution of the groups was M: F 1:1. All the dental pain in this study was attributable to pulpitis due to extensive dental caries of the molar tooth, the duration of pain was 2.9 weeks (range 0.5-8), and the indication for extraction of the non-painful teeth was pericoronitis. All the teeth were removed by standard buccal approach under local or general anaesthesia. Subsequent to the extraction process (lasting less than 5 min), the teeth were sectioned vertically with a water-cooled drill and the pulp lifted out, and specimens immediately snap-frozen at -70°C. Intentional examination of the densely innervated subontoblastic layer of the coronal pulp was assisted by careful orientation of the pulp on a marked sterile card.

**Immunohistochemistry**

Frozen pulp or nerve were embedded in OCT medium (RA Lamb, London, UK) and sections of 8µm thaw-mounted onto glass slides pre-coated with poly-L-lysine. Sections were immersion-fixed in fresh 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min, then endogenous peroxidases blocked by incubation with alcoholic 0.3% hydrogen peroxide for a further 30 min. Sections were incubated overnight with a monoclonal antibody to the neuronal marker neurofilament (Clone 2F11, Dako, Cambridge, U.K., used at a final titre of 1:10, 000) and a polyclonal antibody against the \( \text{Na}_{\text{v}}1.8 \) (K107) [4](Coward et al., 2000). Sites of primary antibody attachment were revealed using avidin-biotin peroxidase method. (Vector Elite ABC method, Vectastain, Novacastria, Newcastle, UK). Preparations were counterstained in 1% w/v aqueous neutral red to visualise nuclei and photographed with an Olympus photomicroscope. Specificity studies of the \( \text{Na}_{\text{v}}1.8 \) antibody (K107),
showing positive staining in human DRG neurons, and no staining in pre-absorption experiments using Na\textsubscript{v}1.8 peptide in tooth pulp sections, were performed as previously described [4].

**Image analysis**

Na\textsubscript{v}1.8 and neurofilament immunoreactivity in fibres were quantified using computerized image analysis (Seescan Cambridge, UK). Images were captured via video link to an Olympus BX50 microscope (×40, objective) and scanned by computer. Setting grey-level detection limits at threshold, highlighted positive immunostaining, and the area of highlighted fibres was obtained as % area of the field scanned. Scanning was performed for a minimum of 5 fields at random per tissue section orientated longitudinally, assessed in a blind fashion. Results are expressed as the average percentage ratio of the mean Na\textsubscript{v}1.8 to neurofilament reactive fibres in 5 fields.

**Analysis**

The Mann Whitney test was used to compare ratios between groups; P values less than 0.05 were considered significant.

**Results**

Immunostaining demonstrated the presence of large numbers of nerve fibres within human tooth pulp that were immunoreactive for neurofilament (Fig. 1A and B). A subset of nerve fibres were also immunostained with the Na\textsubscript{v}1.8 antibody (Fig. 1C and D) in both non-painful and painful pulp groups. Specificity of the Na\textsubscript{v}1.8 antibody was demonstrated in human DRG as positive control with a lack of staining in sampled tissue after the antibody was pre-incubated with peptide.

Using image analysis, there were significantly more fibres immunostaining for Na\textsubscript{v}1.8 in relationship to neurofilament positive fibres in the painful pulp, compared with those without pain (Fig. 2). The median Na\textsubscript{v}1.8 to Neurofilament % area ratio were, for non-painful 0.059 (0.006-0.24) n = 14, for painful 0.265 (0.13-0.5) n = 8, P = 0.0019; fibres detected in non-painful pulp appeared to be mostly localized in discrete trunks, and these trunks were not as apparent in some of the painful specimens. Histologically, there was inflammation of varying severity in several of the painful pulp specimens.

**Discussion**

We have demonstrated for the first time that numerous Na\textsubscript{v}1.8-immunoreactive nerve fibres are present in human dental pulp, in close proximity to the odontoblasts in the subodontoblastic layer, and the Na\textsubscript{v}1.8-immunoreactive fibres were increased in the presence of caries-induced painful pulpitis.

Neurofilament positive fibres have previously been reported in human dental pulp, including fine unmyelinated fibres in the sub odontoblastic layer [12, 14]. Several sensory and sympathetic neuropeptides have been found in dental innervation, including CGRP [15], substance P [16], neuropeptide K [17], neuropeptide Y [18] and vasoactive intestinal polypeptide [19]. The local release of these transmitters may lead to neurogenic inflammation [20]. Sprouting of CGRP positive neurons in the rat dental pulp has been reported after dentine injury [21] and other studies also report an
increase of neuropeptide expression and sprouting in the human pulp [15, 16] infected dental pulp. However, there have been few investigations of the expression of ion channels in the human dental pulp [16].

The presence of Na\textsubscript{v}1.8-immunoreactive neurons identified in the subodontoblastic layer implies that these receptors may be involved in signal transduction at the pulp-dentine junction. It is known that sensory neurones and odontoblasts exist in close proximity, but no synaptic or electrical connections have been identified [11]. Authors in [12] postulated that the odontoblast-neuron connection may be neurochemical. Infection or injury to the pulpal tissues may result in inflammation, resulting in increased expression of substance P, CGRP and collateral nerve sprouting, which are regulated by nerve growth factor (NGF), which also regulates Na\textsubscript{v}1.8 expression by sensory neurons [1]; NGF is itself increased in inflamed pulpal tissues [13]. The sampling of healthy non-painful pulp from partially erupted third molars, though developmentally mature, may not be representative of fully erupted molar pulps. Fibre numbers and receptor expression may change after eruption of the tooth [11]; it remains unknown whether Na\textsubscript{v}1.8-immunoreactivity varies with eruption or maturity of teeth. Although our cross reactivity and pre-absorption studies showed specificity of the antibodies used, the data should be interpreted with caution. Immunostaining of nerves appears to be axoplasmic rather than localised to the nodes of Ranvier and could possibly cross react with other sodium channels. The majority of immunostaining in the dental pulpal samples appeared to be related to small diameter fibres, although some large diameter fibres were immunostained.

Thus while there appeared to be significant differences of the immunolocalisation of Na\textsubscript{v}1.8 between the painful and non-painful samples, there is known heterogeneity of sodium channel expression and other pain receptor expression and a range of modulatory processes aside from NGF regulating Na\textsubscript{v}1.8 expression that must be recognised.

**Conclusions**
In conclusion, nerve fibres in dental pulp from patients with dental pain showed significantly more Na\textsubscript{v}1.8-fibres as a proportion of neurofilament positive fibres. As Na\textsubscript{v}1.8 has been implicated in neuropathic pain, its expression by nerve fibres within human tooth pulp may, contribute to the pathophysiology of dental pain. Further studies of the time-course of the disease, and severity of pain and/or inflammation, are clearly necessary to elucidate the role and regulation of Na\textsubscript{v}1.8 ion channels in the pathophysiology of trigeminal pain, which may represent a target for novel therapeutic agents.

**Competing interests**
None declared
Authors' contributions
TR performed all the surgical procedures, extracted the tooth pulp and helped write the paper. YY participated in the immunohistochemistry, analysis of data and drafted the manuscript. CP, ST and CB were responsible for the design and production of the Na\textsubscript{V}1.8 antibodies used, help with interpretation of the data, and writing the manuscript. CB participated in the conception of the study, development of antibodies, and interpreting the data. PA conceived the study and participated in its design and coordination, interpretation and completion of the manuscript. All authors read and approved the manuscript.

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References


**Figure 1 legend**
Immunoreactive nerve fibres in non-painful (left column) and painful (right column) human tooth pulp sections. Staining with antibodies to neurofilament (A and B), and Na\(_{\text{v}}\)1.8 (C and D). Arrows indicate Na\(_{\text{v}}\)1.8 immunoreactive nerve fibres. Original Magnification x 40

**Figure 2 legend**
Scattergram showing % immunoreactivity of the Na\(_{\text{v}}\)1.8 to Neurofilament ratios in control and painful tooth pulp. The median value is indicated * P<0.05.
Figure 2