International Endodontic Journal

doi:10.1111/j.1365-2591.2011.01853.x

Temporal expression pattern of sodium channel Nav 1.8 messenger RNA in pulpitis

A. Esmaeili[1,2], A. Akhavan[3], M. Bouzari[1], S. B. Mousavi[3], N. Torabinia[4] & S. Adibi[3]

[1]Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran; [2]Department of Biotechnology, Faculty of Advanced Sciences, University of Isfahan, Isfahan, Iran; [3]Torabinejad Dental Research Centre, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran; and [4]Oral Pathology Department, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Esmaeili A, Akhavan A, Bouzari M, Mousavi SB, Torabinia N, Adibi S. Temporal expression pattern of sodium channel Nav 1.8 messenger RNA in pulpitis. *International Endodontic Journal*, **44**, 499–504, 2011.

Aim To determine mRNA expression levels of Nav 1.8 in inflamed pulps of rats.

Methodology Inflammation was induced by creating pulp exposures in rat incisors. Histopathological changes in the induced pulpitis were evaluated 1, 3, 7 and 10 days after exposure. Using real-time PCR, the relative mRNA expression levels of Nav 1.8 in the inflamed rat dental pulp was determined.

Results At day 1, no inflammation was evident in the pulp tissue, whereas increased levels of inflammatory responses were identified at day 3 and day 7. No pulpal inflammation was evident in day 10 or in the control group. Nav 1.8 was expressed in the rat dental pulp and increased at day 3 and day 7. Time course study of dental pulp inflammation indicated that differences in relative mRNA expression levels of Nav 1.8 were correlated with the severity of inflammation.

Conclusions Nav 1.8 channels seem to be expressed significantly more under a temporal control so as to be associated with a severity of inflammation during pulpitis. As Nav 1.8 has been considered to have a role in neuropathic pain, its expression within dental pulp may contribute to the pathophysiology of tooth pain.

Keywords: dental pulp, inflammation, Nav 1.8, rat.

Received 11 March 2010; accepted 20 December 2010

Introduction

In spite of extensive investigation on dental pulp axons, little is known about expression levels of ion channel genes in pulpal tissue. Neural fibre sensitization is likely associated with human pulpal inflammation (Tyldesley & Mumford 1970, Rodd 2000). Alterations in the expression of ion channels including voltage-gated sodium channel isoforms associate with inflammation and may play an important role in neuropathic pain (Amir *et al.* 2006).

Correspondence: A. Esmaeili, Cell, Molecular and Developmental Biology Division, Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran
(Tel.: +96-311732490; fax: +96 311 732456;
e-mail: aesmaeili@sci.ui.ac.ir, abesmaeili@yahoo.com).

The tetrodotoxin-resistant sodium channel Nav 1.8 with rapid activation and slow inactivation belongs to the voltage-gated ion channel superfamily and is highly expressed in small-diameter sensory neurons (Renganathan *et al.* 2001, Lai *et al.* 2004). These channels are expressed in nociceptive neurons of the dorsal root ganglions (Dib-Hajj 2007). Direct nerve injury leads to immunocytochemical and electrophysiological changes in Nav 1.8 channels suggesting a potential role in the sensation of neuropathic pain (Gold *et al.* 2003). Patients with chronic neurogenic pain or chronic local hyperalgesia also show increased Nav 1.8 channel expression proximal to a peripheral injury site (Coward *et al.* 2000, 2001, Yiangou *et al.* 2000).

Presence of nerves containing Nav 1.8 in layers of the pulp subodontoblastic region may reveal an important role for sodium channels in dental pain.

Immunohistopatholgy has also shown the presence of Nav 1.8 in the dental pulp (Renton *et al.* 2005). The expression level of Nav 1.8 is regulated by sensory neurons. One of the factors that may affect expression levels of Nav 1.8 is changing pulp tissue, specifically inflammation. Injury to dental pulp tissue causes inflammation that can lead to pain (Waxman 1999). Changes in sensory neurons may associate with chronic dental pain (Djouhri *et al.* 2003a,b). Nerve fibres in pulp tissue from patients with dental pain showed significantly more Nav 1.8 channels compared to a proportion of neurofilament positive fibres (Renton *et al.* 2005). As Nav 1.8 has been implicated in neuropathic pain, its expression by nerve fibres within human tooth pulp tissue may contribute to the pathophysiology of dental pain. The effects of inflammation severity on mRNA expression of Nav 1.8 have not investigated. Therefore, this study aimed to determine mRNA expression levels of Nav 1.8 according to the time-course and the severity of inflammation. To attain this goal real time RT-PCR as a sensitive quantitative method was used. Results of the current study may elucidate the role and regulation of Nav 1.8 ion channels in the pathophysiology of trigeminal pain.

Materials and methods

Animals

Sixty adult male Wistar rats weighing between 180 and 240 g at the beginning of experiment were used. The rats were assigned to five groups (12 rats in each group): a nontreatment group (control group); four inflammation groups (day 1, day 3, day 7 and day 10). They were housed under a 12:12-h light/dark cycle with *ad libitum* access to food and water. All experiments were approved by the Animal Ethics Committee for the University of Isfahan and Isfahan University of Medical Sciences, Iran.

Experimental dental pulp inflammation

For inducing injury in incisor pulp tissue, the animals were anaesthetized with a combination of isofluran and nitrous oxide inhalation. Body temperature was maintained at 37 °C during surgery using a heating pad. To induce pulpal exposures right mandibular and maxillary incisors at the gingival margin of the teeth were decoronated using a high-speed size 10 coarse diamond bur (D&Z, Berlin, Germany). For each case a new diamond bur was used. The coronal root canal was enlarged with hand reamers (size 25, GC, Tokyo, Japan) and the cavity left open.

Pulp extirpation

Control rats were sacrificed at 0 h (non-treated), and treated rats were sacrificed at 1, 3, 7 and 10 days by decapitation under light ether anaesthesia. The maxillary and mandibular jaws were removed from rats and the incisors were extracted. Half of the teeth (both mandibular and maxillary incisors) were used for reverse-transcription/polymerase chain-reaction (RT-PCR), and half were used for histological studies. Dental pulp tissue was removed from the pulp chamber and extirpated with a stainless steel barbed broach (size 20) (VDW, Munich, Germany) from the pulp stumps. The pulp tissues were carefully extirpated cut into several pieces and then kept at −70 °C until tested.

Histological study

The extracted teeth were immediately fixed with 10% formalin (pH 7.4) for 3 days, decalcified for 48 h with 10% nitric acid at room temperature. All regions of associated soft tissues then were serially sectioned at 5-μm thickness, stained with haematoxylin & eosin and examined under a light microscope. The inflammatory response of pulp tissues were scored according to predefined criteria (Aeinehchi *et al.* 2003, Ooms *et al.* 2003) (Table 1) in high-power fields (×400). The scoring of the tissues was conducted by the same examiner throughout.

Real time PCR analysis

Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's specifications. Total RNA was assessed by optical density measurements. DNase treated total RNA (1 μg) was reverse transcribed using QuantiTect RT kit (Qiagen) according to the manufacturer's instructions.

PCR reactions were performed in a 25 μL volume containing 12.5 μL iQ SYBR Green supermix kit (BioRad, Hercules, CA, USA), 10 pM of specific Nav 1.8 and 18S (as housekeeping gene) forward and reverse primers with 5 ng of template. Samples were made up to 25 μL with RNase–DNasefree water. Reactions were carried out on a Chromo 4 System Instrument (Bio-Rad) PCR machine as follows: an initial 95 °C denaturation for 10 min and 50 cycles of amplification reactions at 95 °C for 30 s, 58 °C for

1 min and 72 °C for 30 s, followed by melting curve analysis and cooling process. The melting curve analysis was initiated at 55 °C with elevation of the temperature up to 95 °C at a heating rate of 0.1 °C s^{-1}. The conditions were identical for Nav 1.8 and 18S. The primers used for real time PCR were:

Nav 1.8: AAGGGACAGGAGCAGTTG/CAGGTATGGAGCCAGGTC.

Expression levels were normalized to that of the constitutive 18S ribosomal RNA with primers:

18S: GTAACCCGTTGAACCCCATT/CCATCCAATCGGTAGTAGCG.

Relative expression data were quantified using $2^{-(\text{Ct sample-Ct control})}$ where Ct is the cycle threshold. Relative standard curves were generated by plotting the threshold value (Ct) versus the log of the amount of total cDNA added to the reaction and used to check the efficiency of primers. Calculation of Ct, standard curve preparation and quantification of mRNA in the samples were performed using Excel (Microsoft Office Excel 2007, Redmond, CA, USA).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software package (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was determined by Tukey one-way ANOVA. A P value of less than 0.05 was chosen as the significance level for all statistical analyses. The Spearman coefficient was also used. All data are presented as the mean ± standard error (SEM).

Results

Histological findings for experimentally induced rat pulpitis

Based on light microscopic observations, only inflammation scores of 0–2 were observed in the tissues. Visible differences were obvious in the degree or extent of the pulpal inflammation between the control and experimental animals. At day 1, the median value of inflammation was 0 in the root canal (Fig. 1a). However, increased levels of inflammatory response (1) were detected at day 3 (Fig. 1b). Pulp exposures induced a strong inflammatory reaction (2) at day 7 (Fig. 1c). These pathological findings were characterized by infiltration of inflammatory cells, the dilation of blood vessels and focal areas of necrosis. No pulpal inflammation was evident at day 10.

Real time RT-PCR findings

To identify levels of Nav 1.8 present in the dental pulp mRNA expression of this ion channel was examined in a normal incisor tooth by real time RT-PCR. Nav 1.8 was expressed in this normal tooth. Next the expression levels of Nav 1.8 were determined during the progression of inflammation. Real-time PCR profiles (Fig. 2) showed an enhancement in mRNA expression levels of Nav 1.8 from day 1 to day 7. The highest level of expression was seen at day 7. From day 7 to day 10 a significant reduction in expression levels was observed (Fig. 2). The relationship between the observed inflammation and gene expression was determined. Using the Spearman coefficient, a significant correlation between the Nav 1.8 expression and pulpal inflammation was observed. The correlation coefficient value was 0.90 ($P = 0.035$), indicating a strong relationship.

Discussion

The role of ion channels, receptors and signalling mechanisms in pain pathophysiology, in which specific channels play a role in neuropathic and inflammatory pain, have been studied extensively (Rogers *et al.* 2006, Zhao *et al.* 2008, Siqueira *et al.* 2009, Thakor *et al.* 2009). Because of pain disorders or unresponsiveness to pain, studying the role of voltage-gated sodium channels in chronic pain has recently gained interest

Table 1 Criteria for scoring of inflammatory tissue responses

No inflammation (grade 0)	Mild Inflammation (grade 1)	Moderate inflammation (grade 2)	Severe inflammation (grade 3)
No inflammatory cells More than 30 fibroblasts Mature fibrous tissue with many collagens.	Presence of macrophages and/or plasma cells. Less than 30 inflammatory cells. 10–30 Fibroblasts. Immature fibrous tissue with little collagen.	Presence of macrophages and plasma cells. Occasional foci of neutrophils, granulocytes and/or lymphocytes. More than 30 and less than 60 inflammatory cells. 5–9 Fibroblasts	Focal areas of necrosis. Tissue densely infiltrated by inflammatory cells. More than 60 inflammatory cells. 1–4 Fibroblasts.

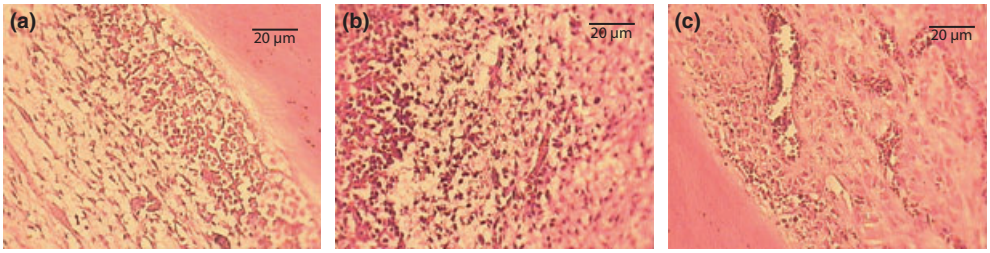

Figure 1 Degree of rat dental pulp inflammation after exposure. Figures show examples of the different inflammation grades. Grade 0, grade 1 and grade 2 respectively (a–c). Haematoxylin–eosin stain, original magnification ×400.

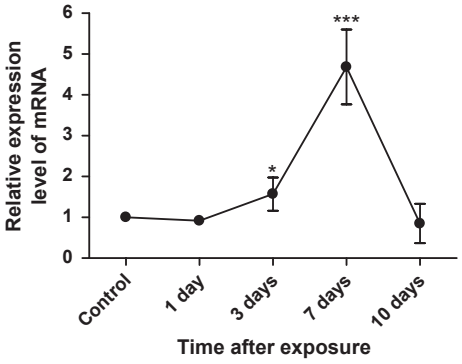

Figure 2 Quantitative analysis of Nav 1.8 mRNA expression in dental pulp tissue after injury. Nav 1.8 mRNA increased significantly between 3 and 7 days after injury then gradually declined. Significant difference ($^{*}P < 0.05$, $^{***}P < 0.001$, Tukey *post hoc* test) compared to the sham operated group.

(Cox *et al.* 2006, Ahmad *et al.* 2007, Goldberg *et al.* 2007). Nav 1.3, Nav 1.7, and Nav 1.8 subtypes are expressed in many chronic pain conditions (Rogers *et al.* 2006, Rush *et al.* 2006, Cummins *et al.* 2007, Dib-Hajj *et al.* 2007). In the present study, the expression of Nav 1.8 in inflamed dental pulp was examined and compared to a control group. The effects of the degrees of inflammation on expression of this channel 1, 3, 7 and 10 days after induction of injury was also determined.

In neuropathic and inflammatory pain the expression and activity of several voltage-gated sodium, potassium, and calcium channels may change. The expression pattern of these ion channels within specific tissues may either be up- or down-regulated after nerve injury. A variety of tetrodotoxin-sensitive (Nav 1.3 and Nav 1.7) and tetrodotoxin-insensitive (Nav 1.8 and Nav 1.9) channels are involved in regulating sensory neural excitability (Matzner & Devor 1994, Eglen *et al.* 1999). In acute inflammatory pain, Nav 1.7 and Nav 1.8 are overexpressed (Cummins *et al.* 2007, Dib-Hajj *et al.* 2007). The expression of Nav 1.3, Nav 1.7 and Nav 1.8 by RT-PCR in patients with trigeminal neuralgia an idiopathic paroxysmal pain showed changes in their expression (Siqueira *et al.* 2009). Changes in the expression, trafficking, and redistribution of sodium channels, after inflammation or nerve injury, are considered to account for unstable oscillations of membrane potential, abnormal firing, and the generation of ectopic activity in afferent nerves (Rogers *et al.* 2006).

Prostaglandins, bradykinin, ions, and the release of neuronal growth factors (NGF), increase the expression of sodium channels, and characterize primary hyperalgesia.

At the dorsal horn or trigeminal nuclei system, the upregulation of voltage-gated sodium channels is involved in secondary hyperalgesia and central sensitization, with long-term potentiation, higher strength in synaptic connections and memory of pain (Sandkühler, 2007). The presence of Nav 1.8 in pulp tissue has previously been shown (Renton 2005, Warren *et al.* 2008, Luo *et al.* 2008). In some studies, Nav 1.3 remains upregulated, (Cummins *et al.* 2007, Dib-Hajj *et al.* 2007, Siqueira *et al.* 2009); in other studies of neuropathic mechanisms, Nav 1.7 and Nav 1.8 are downregulated (Cummins *et al.* 2007, Dib-Hajj *et al.* 2007). However, in the present study, Nav 1.8 had an increased expression when compared to controls. The underlying hypotheses of these findings are discussed below. Evidence provided in the present paper and in previous studies (Renton 2005, Henry *et al.* 2009) demonstrates that Nav 1.8 is expressed in pulp tissue, however the physiological role of these receptors is not known.

Inflamed pulps compared to controls showed upregulation of Nav 1.8 at day 3 and 7. There was a correlation between the expression of Nav 1.8 and degree of inflammation; in other words that higher

expression of Nav 1.8 that occurred in pulps was associated with a higher degree of inflammation.

Nav 1.8 is expressed in small sensory neurons and may be down-regulated in small injured axons but up-regulated in adjacent uninjured C fibres in human peripheral neuropathies (Coward *et al.* 2000, Gold *et al.* 2003). Nav 1.8 appears to be important for the generation of spontaneous activity in damaged sensory axons (Roza *et al.* 2003), and knock down of Nav 1.8 in models of neuropathic pain produces a marked reduction in abnormal pain responsiveness (Gogas 2006). It has been demonstrated that Nav 1.8 has a greater involvement in frank nerve injury and inflammatory pain as compared to acute, post-operative or chemotherapy-induced neuropathic pain states (Joshi *et al.* 2006). The results of the present study are in agreement to these previous findings which determined Nav 1.8 expression in dental pulp nerves.

While it has traditionally been thought that Nav 1.8 mRNA, and other mRNAs, are restricted to the cell body of sensory neurons, recent evidences suggest that this might not be the case. Enhancement in Nav 1.8 mRNA may be due to its trafficking to the periphery after injury to the tooth. It is shown that sodium channel mRNA could be peripherally transported from the DRG to the sciatic nerve and locally translated (Thakor *et al.* 2009). Recently the role of local protein synthesis in sensory neurons and its contribution to nociception were reviewed (Price & Geranton 2009). Some experiments have shown recently that Nav 1.8 and other sodium channels are expressed by cells such as epidermal keratinocytes and machrophages (Craner *et al.* 2005, Carrithers *et al.* 2007, Zhao *et al.* 2008). This may also explain the presence and up-regulation of Nav 1.8 mRNA within the tooth pulp after injury in this study. It is anticipated that determining Nav 1.8 expressed in dental pulp will facilitate future research into the role of these receptors using an increasingly available subtype-specific pharmacology.

Conclusion

Inflamed rat dental pulp tissue had significantly higher expression levels of Nav 1.8. As Nav 1.8 has a role in neuropathic pain, its expression within human dental pulp tissue may contribute to the pathophysiology of tooth pain. Further studies of the time-course of severity of pain and/or inflammation in human are necessary to clarify the role of Nav 1.8 ion channels in the pathophysiology of trigeminal pain. Also investigations into the effects of Nav 1.8 agonists and antagonists as novel analgesic agents are needed. One of the future challenges in the development of novel sodium channel blockers is to design and synthesize isoform-selective channel inhibitors.

Acknowledgements

This study was supported by grants from the Isfahan University of Medical Sciences (Grant no. 185265).

References

Aeinehchi M, Eslami B, Ghanbariha M, Saffar AS (2003) Mineral trioxide aggregate (MTA) and calcium hydroxide as pulp-capping agents in human teeth: a preliminary report. *International Endodontic Journal* **36**, 225–31.

Amir R, Argoff CE, Bennett GJ, Cummins TR (2006) The role of sodium channels in chronic inflammatory and neuropathic pain. *Journal of Pain* **7**, S1–29.

Carrithers MD, Dib-Hajj S, Carrithers LM *et al.* (2007) Expression of the voltage-gated sodium channel NaV1.5 in the macrophage late endosome regulates endosomal acidification. *Journal of Immunology* **178**, 7822–32.

Coward K, Plumpton C, Facer P *et al.* (2000) Immunolocalization of SNS/PN3 and NaN/SNS2 sodium channels in human pain states. *Pain* **85**, 41–50.

Coward K, Jowett A, Plumpton C *et al.* (2001) Sodium channel beta 1 and beta 2 subunits parallel SNS/PN3 alpha-subunit changes in injured human sensory neurons. *Neuroreport* **12**, 483–8.

Craner MJ, Damarjian TG, Liu S *et al.* (2005) Sodium channels contribute to microglia/macrophage activation and function in EAE and MS. *Glia* **49**, 220–9.

Dib-Hajj SD (2007) Ion channel pathophysiology as a molecular basis for pain. *Journal of the Peripheral Nervous System* **12**, 1.

Djouhri L, Fang X, Okuse K, Wood J, Berry C, Lawson S (2003a) The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. *Journal of Physiology* **550**, 739–52.

Djouhri L, Newton R, Levinson S, Berry C, Carruthers B, Lawson S (2003b) Sensory and electrophysiological properties of guineapig sensory neurones expressing Nav 1.7 (PN1) Na+ channel alpha subunit protein. *Journal of Physiology* **546**, 565–76.

Eglen RM, Hunter JC, Dray A (1999) Ions in the fire: recent ion-channel research and approaches to pain therapy. *Trends in Pharmacological Sciences* **20**, 337–42.

Gogas KR (2006) Glutamate-based therapeutic approaches: NR2B receptor antagonists. *Current Opinion in Pharmacology* **6**, 68–74.

Gold MS, Weinreich D, Kim CS *et al.* (2003) Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain. *Journal of Neuroscience* **23**, 158–66.

Henry MA, Luo SJ, Foley BD, Rzasa RS, Johnson LR, Levinson SR (2009) Sodium channel expression and localization at demyelinated sites in painful human dental pulp. *Journal of Pain* **10**, 750–8.

Joshi SK, Mikusa JP, Hernandez G *et al.* (2006) Involvement of the TTX-resistant sodium channel Nav 1.8 in inflammatory and neuropathic, but not post-operative, pain states. *Pain* **123**, 75–82.

Lai J, Porreca F, Hunter J, Gold M (2004) Voltage-gated sodium channels and hyperalgesia. *Annual Review of Pharmacology and Toxicology* **44**, 371–97.

Matzner O, Devor M (1994) Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na+ channels. *Journal of Neurophysiology* **72**, 349–59.

Ooms EM, Egglezos EA, Wolke JG, Jansen JA (2003) Soft-tissue response to injectable calcium phosphate cements. *Biomaterials* **24**, 749–57.

Price TJ, Geranton SM (2009) Translating nociceptor sensitivity: the role of axonal protein synthesis in nociceptor physiology. *European Journal of Neuroscience* **29**, 2253–63.

Renganathan M, Cummins TR, Waxman SG (2001) Contribution of Na(v)1.8 sodium channels to action potential electrogenesis in DRG neurons. *Journal of Neurophysiology* **86**, 629–40.

Renton TY, Yiangou C, Plumpton S, Tate C, Bountra P, Anand (2005) Sodium channel Nav1.8 immunoreactivity in painful human dental Pulp. *BMC, Oral Health* **5**, 5.

Rodd HB, Boissonade FM (2000) Substance P expression in human tooth pulp in relation to caries and pain experience. *European Journal of Oral Sciences* **108**, 467–74.

Rogers M, Tang L, Madge DJ, Stevens EB (2006) The role of sodium channels in neuropathic pain. *Seminars in Cell & Developmental Biology* **17**, 571–81.

Roza C, Laird JMA, Souslova V, Wood JN, Cervero F (2003) The tetrodotoxin-resistant Na+ channel Nav1.8 is essential for the expression of spontaneous activity in damaged sensory axons of mice. *Journal of Physiology-London* **550**, 921–6.

Siqueira SRDT, Alves B, Malpartida HMG, Teixera MJ, Siqueira JTT (2009) Abnormal expression of voltage-gated sodium channels Nav 1.7, Nav 1.3 and Nav 1.8 in trigeminal neuralgia. *Neuroscience Letters* **164**, 573–7.

Thakor DK, Lin A, Matsuka Y *et al.* (2009) Increased peripheral nerve excitability and local NaV1.8 mRNA up-regulation in painful neuropathy. *Molecular Pain* **5**, 14.

Tyldesley W, Mumford J (1970) Dental pain and the histological condition of the pulp. *Dent Praet Dent Ree* **20**, 333–6.

Warren CA, Mok L, Gordon S, Fouad AF, Gold MS (2008) Quantification of neural protein in extirpated tooth pulp. *Journal of Endodontics* **34**, 7–10.

Waxman S (1999) The molecular pathophysiology of pain: abnormal expression of sodium channel genes and its contributions to hyperexcitability of primary sensory neurons. *Pain* SuppI6S, 133–40.

Yiangou Y, Birch R, Sangameswaran L, Eglen R, Anand P (2000) SNS/PN3 and SNS2/NaN sodium channel-like immunoreactivity in human adult and neonate injured sensory nerves. *Febs Letters* **467**, 249–52.

Zhao P, Barr TP, Hou QZ *et al.* (2008) Voltage-gated sodium channel expression in rat and human epidermal keratinocytes: Evidence for a role in pain. *Pain* **139**, 90–105.

