Voltage-Gated Sodium Channel Expression in Rat Spiral Ganglion Neurons

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Abstract

The spiral ganglion neurons (SGN) provide the afferent innervation of the hair cells in the organ of Corti and relay auditory information from the inner ear to the brain. Voltage-gated sodium channels (Nav) initiate and propagate action potentials that encode this sensory information but little is known regarding the subtypes expressed in these cells. We have used RT-PCR and immunohistochemistry to study the complement and anatomical distribution of Nav channels in rodent SGN. Nav1.1, Nav1.6 and Nav1.7 were all detected at the mRNA level. Fluorescence or streptavidin-horseradish peroxidase immunohistochemistry extended these findings, demonstrating predominant localisation of Nav1.6 and Nav1.7 on SGN cell bodies and Nav1.1 on axonal processes. Dual labelling with peripherin demonstrated higher Nav1.6 and Nav1.7 expression on Type I, rather than Type II neurons. These results provide evidence for selective expression and variations in the distribution of VGSC in the rodent SGN, which may guide further studies into afferent function in the auditory pathway and therapeutic approaches for diseases such as hearing loss and tinnitus.

Keywords: Cochlea; immunohistochemistry; peripherin; RT-PCR; spiral ganglion neurons; voltage-gated sodium channels

Introduction

There are nine recognised members of the voltage-gated sodium channel family (VGSC; Nav1.1-1.9). Of these Nav1.1, 1.2, 1.3, and 1.6 are highly (but not exclusively) expressed in the central nervous system (Westenbroek, Merrick and Catterall, 1989, Beckh et al., 1989, Whitaker et al., 2000, Whitaker et al., 2001, Tzoumaka et al., 2000) whereas Nav1.7, 1.8 and 1.9 demonstrate a more restricted expression pattern, in autonomic and sensory neurons in the peripheral nervous system (Sangameswaran et al., 1997, Toledo-Aral et al., 1997, Sangameswaran et al., 1996, Dib-Hajj et al., 1998a), and Nav1.4 and Nav1.5 represent the predominant skeletal muscle and cardiac sodium channels, respectively (Trimmer et al., 1990, Rogart et al., 1989). The Nav1.6 sub-type is also highly expressed in the peripheral nervous system where it is enriched at the nodes of Ranvier of myelinated axons and contributes to saltatory conduction (Caldwell et al., 2000, Krzemien et al., 2000).

The spontaneously active spiral ganglion neurons (SGN) form the afferent conduction pathway in the auditory periphery. There are two types of SGN, Type I neurons that innervate the inner hair cells and Type II neurons that innervate the outer hair cells (Dallos, 1992). The conduction of nerve impulses along these afferents is controlled by a number of ion channels including the voltage-gated sodium channels. Clearly, establishing the expression and distribution of the sodium channels expressed in the auditory periphery could impact our understanding of action potential dynamics and coding of auditory stimuli as well as normal and abnormal phenotypes (Patuzzi et al., 2004, Hossain et al., 2005). This knowledge would also contribute to the development of putative therapeutics for disorders such as tinnitus, enabling more precise targeting of channels involved in tinnitogenesis and, potentially provide opportunities for the rational development of sub-type selective ligands to address this area of unmet need (Vio and Holme, 2005). To date, only one study has been reported

that has examined VGSC expression and distribution in SGN (Hossain *et al.*, 2005) but this was limited to Nav1.2, and Nav1.6. The aim of this study, therefore, was to fully characterise the expression and distribution pattern of relevant VGSCs using RT-PCR and Nav subtype selective antibodies in rat SGN.

Results

Na_v alpha subunit expression using RT-PCR

Validation of primer specificity in Rat cerebral cortex and DRG: To confirm the specificity of the primers used, tissue with previously documented Na_v α subunit expression were utilised. Distinct bands at the correct predicted sizes were observed for Na_v1.1, Na_v1.2, Na_v1.3 and Na_v1.6 in the cerebral cortex, with weak bands present for Na_v1.7 and Na_v1.9 (Fig. 1A). With the mRNA isolated from the rat DRG, distinct bands were seen for Na_v1.1, Na_v1.7, Na_v1.8 and Na_v1.9 while a weak band was observed for Na_v1.6 (Fig. 1B).

Rat spiral ganglion: The RT-PCR primers specific for the Na_v α subunits and β -actin were used to probe the cDNA created from mRNA isolated from rat modioli homogenates. Distinct bands at the correct predicted molecular weight were observed for Na_v1.7, Na_v1.6 and Na_v1.1, as well as the control gene β -actin (Fig. 1C). No bands were seen in the lane without reverse transcription, showing that there was no genomic DNA contamination.

Immunocytochemical localization of Na_v alpha subunits in spiral ganglion neurons

Na_v1.7: The Na_v1.7 antibody strongly labelled the cell bodies of rat SGN using fluorescently tagged secondary antibodies (Fig. 2A and B). Labelling was found throughout the cytoplasm of the cell body, with no clear differentiation between membrane and cytoplasmic labelling. Variation was observed in the intensity of labelling, with some neurons strongly labelled and others only faintly labelled (Fig. 2B).

In sections of rat cochlea incubated without the primary antibody, the labelling was reduced to background levels although pale yellow puncta were noted throughout the cytoplasm (Fig. 2C).

To demonstrate that this punctate labelling was due to non-specific fluorescence, experiments were repeated using the same Nav1.7 antibody but with streptavidin/horseradish peroxidase (HRP) and DAB to non-fluorescently visualise the primary antibody binding. Using this method, the SGN were positively labelled with brown reaction product as seen in Figs 2D and E. The positively labelled SGN cell bodies showed variation in staining intensity, similar in appearance to that observed in the fluorescence imaging technique. Additionally, the sections of rat cochlea incubated without the primary antibody did not show neurons labelled with the brown reaction product, nor the punctate labelling observed in the previous controls using fluorescently tagged antibodies (Fig. 2F). This confirmed that the puncta could be considered as tissue autofluorescence.

Nav1.6: The Nav1.6 antibody strongly labelled the cell bodies of rat SGN (Fig. 3A and B). The pattern of distribution was similar to that seen with the Nav1.7 antibody. Labelling was found throughout the cytoplasm of the cell body, with no clear differentiation between membrane and cytoplasmic labelling. Additionally, there was again considerable variation observed in the intensity of neuronal labelling as seen in Fig. 3B. In contrast to the labelling seen with Nav1.7, peripheral and central processes were also strongly labelled for Nav1.6 α -subunits

As performed previously, sections of rat cochlea incubated without the primary antibody the labelling was reduced to background levels, although pale yellow autofluorescent puncta were observed throughout the cytoplasm as were also seen for Nav1.7 (Fig. 3C). As for Nav1.7, this punctate labelling was demonstrated as non specific by using the HRP/DAB method to visualise non-fluorescently primary antibody binding. The SGN were positively stained for Nav1.6 with the brown reaction product using this method as seen in Figs 3D and

E. Again, positively labelled SGN cell bodies showed variation in staining intensity similar in appearance to that seen with fluorescence imaging. The distinct staining of the neuronal processes was replicated and, in addition, staining of the cell body membranes was observed (Fig. 3E). Sections of rat cochlea incubated without the primary antibody did not show neurons labelled with the brown reaction product nor the punctate labelling observed in the previous fluorescence controls (Fig. 3F).

Nav1.1: The Nav_v1.1 antibody weakly stained the SG neurons and was only detectable using the non-fluorescent HRP/DAB method as seen in Figure 4. Weak staining was observed in the SGN cell bodies while darker staining was seen in the neuronal processes, specifically in the axons (Fig 4A and B). The sections of rat cochlea incubated without the primary antibody did not show labelled neurons (Fig. 4C).

Peripherin labelling of spiral ganglion neurons

It has been previously reported that antibodies generated against the intermediate filament protein, peripherin, preferentially label Type II SGN in sections of cochlea (Hafidi, 1998, Mou, Adamson and Davis, 1998, Reid, Flores-Otero and Davis, 2004). A small proportion (33/242 or $\approx 14\%$) of SGN were labelled by this antibody. Most noticeably, all the labelled neurons appeared to be smaller compared to the unlabelled neurons (Fig. 5A).

Peripherin labelling status and Nav1.7: The relationship between cell body area and peripherin labelling status was investigated further by dual labelling sections of cochlea with peripherin and Nav_v1.7 antibodies. This was performed using sections from four cochleae (Fig 5A and B). The frequency histogram of cell body area in Fig. 5C shows that distribution in peripherin positive neurons was distinctly separate from that of peripherin negative neurons.

The mean difference in peripherin negative and positive cell body areas was significantly different, from $165.3 \mu\text{m}^2 \pm 1.8$ (n=209) vs $82.2 \mu\text{m}^2 \pm 2.6$ (n=33) respectively, $p < 0.0001$. This represents about a two-fold difference in cell body area.

On visual inspection of the sections it appeared that the peripherin positive neurons were labelled less intensely by the $\text{Na}_v1.7$ antibody than the peripherin negative neurons. To investigate the relationship between peripherin and density of $\text{Na}_v1.7$ labelling further, the average fluorescence intensity of the neurons labelled with the $\text{Na}_v1.7$ antibody and peripherin negative and positive neurons was measured in four cochleae.

The mean $\text{Na}_v1.7$ fluorescence was significantly different between the peripherin negative and positive neurons, from 120.1 ± 1.4 vs 100.2 ± 3.7 respectively at $p < 0.01$. This confirmed the impression of differences in staining intensity between the two groups. Combination of these two parameters in a scatter plot of cell body area versus average fluorescence in Fig 5E, further illustrates that peripherin negative neurons possess larger cell body areas and label more strongly with $\text{Na}_v1.7$ antibody.

Peripherin labelling status and $\text{Na}_v1.6$: An identical set of dual labelling experiments to those with $\text{Na}_v1.7$ were performed, using peripherin and $\text{Na}_v1.6$ antibodies and sections from the same rat cochleae that were used previously. Similar initial observations and results with regards to antibody staining, cell body area and fluorescence intensity were obtained as shown in Figs 6 A-E. With $\text{Na}_v1.6$ the difference in distribution and means of cell body area between peripherin negative and positive was more marked than for $\text{Na}_v1.7$. The differences in mean cell body area were significantly different with means of $185.7 \pm 2.2 \mu\text{m}^2$ (n=203) vs $74.8 \pm 3.2 \mu\text{m}^2$ (n=34) respectively, $p < 0.001$, or about a 2.5 fold difference in cell body area.

Differences in mean Na_v1.6 fluorescence between the negative and positive stained peripherin neurons were also significantly different, with mean difference of about 40 units; 142.6 ± 1.7 (n=203) vs 101.6 ± 3.2 (n=34) respectively, $p < 0.001$.

Combination of the two parameters in a scatter plot of cell body area versus average fluorescence in Fig 5E, further illustrates that peripherin negative neurons label more intensely than with Na_v1.6 antibody.

Comparison of Na_v1.6 and Na_v1.7 SGN labelling: Direct comparison of Na_v1.6 and Na_v1.7 immunofluorescence values was not justified as the relative antibody binding affinities were not known. However, SGN cell body area could be compared directly. The Type I means of $185.7 \pm 2.2 \mu\text{m}^2$ vs $165.3 \mu\text{m}^2 \pm 1.8 \mu\text{m}^2$ for Na_v1.6 and Na_v1.7 respectively, were highly significantly different at $p < 0.001$. The respective difference in Type II means, $74.8 \pm 3.2 \mu\text{m}^2$ vs $82.2 \mu\text{m}^2 \pm 2.6$, for Na_v1.6 and Na_v1.7 was not significant at $p > 0.05$.

Peripherin labelling status and Na_v1.1: Dual labelling experiments with peripherin and Na_v1.1 antibodies could not be performed because of the weak labelling of the SGN cell bodies using Na_v1.1 antibodies.

Discussion

Rat SGN exhibits a unique pattern of Na_v subunit expression: The results presented here provide for the first time strong evidence that the α -subunits, Na_v1.7, Na_v1.6 and Na_v1.1, are selectively expressed in adult rat SGNs. There are a number of unusual features in this pattern of Na_v subunit expression, as they suggest that expression in the SGN does not follow that observed in either the central or peripheral nervous system. Instead, the results presented here show Na_v isoforms normally considered to be specific to the peripheral and central nervous system are both expressed in the SGN. The explanation for this may be related to the independent embryological origins of the SGN (Fekete and Wu, 2002). The peripheral and central nervous systems are known to arise from different progenitor cells in the developing embryo. The CNS forms from the dorsal ectoderm through invagination and formation of the neural tube. DRG neurons arise from neural crest cells that migrate laterally to form the peripheral ganglia (Kuan et al., 2004, Li, Say and Zhou, 2007). In contrast, the SGN develops from a distinct region of neural ectoderm known as the otic placode, which is separate from both the neural tube and neural crest cells (Holley, 2005, Fekete and Wu, 2002). The results from this study suggest that the otic placode displays an intermediate phenotype with regard to Na_v channel expression between the CNS and PNS.

Na_v1.1 subunit expression: Na_v1.1 has been previously been shown to be strongly expressed by neurons in the central nervous system (Westenbroek, Merrick and Catterall, 1989). The preliminary results from this study appear to show that Na_v1.1 is weakly expressed in the SGN with strong axonal localisation. As dual labelling experiments could not be performed using the Na_v1.1 antibody, we cannot report if Na_v1.1 expression is restricted to the Type I or Type II neurons.

Nav1.7 subunit expression: Nav1.7 is considered to be exclusively expressed by neurons in the PNS, especially in the cell bodies (Sangameswaran et al., 1997) and peripheral terminals or growth cones (Toledo-Aral et al., 1997) of DRG neurons. The results presented here also show that the Nav1.7 subtype is expressed in the cell bodies of the rat SGN. In contrast to this rodent model, Nav1.7 was not reported to be present in the murine model of Hossain *et al.*, (2005). This may reflect a species difference in expression of the Nav1.7 subunit.

Nav1.6 subunit expression: It has been previously reported that the Nav1.6 subtype is expressed in murine SGN (Hossain et al., 2005) with Nav1.6 localised at the initial segment and nodes of Ranvier and not in the cell bodies. The results here in the rat SGN are also partly in line with this earlier study. Nav1.6 labelling was found in the cell body cytoplasm as well as the processes of the rat SGN. The presence of cytoplasmic labelling in the cell body seen here is in agreement with previous reports of Nav1.6 labelling in other sensory ganglia (Tzoumaka et al., 2000, Krzemien et al., 2000, Black et al., 1999). In addition, cytoplasmic labelling has also been reported for potassium channels in the cell bodies of murine SGN (Adamson et al., 2002) and rat DRG neurons (Rasband et al., 2001). This type of labelling is most likely due to the pooling of subunit proteins in the Golgi apparatus, prior to their transport and insertion into the membrane.

Differential expression of Nav1.6 and Nav1.7 in SGN Type I and Type II: The measurement of SGN cell body area along with semi-quantitative fluorescence and non-fluorescence imaging techniques enabled the evident variation in labelling intensity to be explored in more detail. Resolution of differential expression was further enhanced by the use of dual labelling with peripherin antibody (Hafidi, 1998, Mou, Adamson and Davis, 1998, Reid, Flores-Otero

and Davis, 2004). The differences in SGN Type 1 and II cell body area have been reported previously and are confirmed by this study (Berglund and Ryugo, 1987).

Whilst reduced fluorescence for both $\text{Na}_v1.6$ and 1.7 was apparent in Type II neurons, the intensity of labelling observed was above background levels, indicating that Type II SGN express these subunits. This would support their possessing some functional capacity. Currently, little electrophysiological evidence exists to identify the specific role of Type II fibres or that they actually exhibit any acoustically driven activity (Brown, 1994, Robertson, Sellick and Patuzzi, 1999), although it has been proposed they may signal static displacement of the basilar membrane (Robertson, Sellick and Patuzzi, 1999).

The difference in cell area between $\text{Na}_v1.6$ and 1.7 expressing Type I neurons may be explained in at least two ways. One hypothesis is that the difference may correlate with signalling function as spontaneous activity in mammalian Type I afferents is known to range between $0\text{-}120 \text{ spikes sec}^{-1}$, which correlates with axon diameter (Lieberman, 1982). It would be reasonable to assume that axon diameter also correlates with cell body area and that the expression of $\text{Na}_v1.6$ and 1.7 in Type I SGN may then be related to the spontaneous and/or driven activity of Type I fibres. Alternatively, the differences may simply reflect more intense membranous staining by $\text{Na}_v1.6$ over 1.7 . $\text{Na}_v1.6$ has a role in action potential initiation and propagation in many neurons due to its localisation at the axon hillock and nodes of Ranvier, respectively. By contrast $\text{Na}_v1.7$, a channel that shows slow inactivation and stays open in response to slow ramp depolarisations, may have a role in initiating action potential initiation at the hair cell-afferent fibre synapse.

Expression of other Nav isoforms in the SGN: The expression of other voltage-gated sodium channel isoforms was also investigated in this study. RT-PCR failed to show the expression of mRNA for Nav1.2, Nav1.3, Nav1.8 and Nav1.9, indicating that these isoforms are not expressed in the rat SGN.

Interestingly, in the murine study by Hossain *et al.*, (2005), Nav1.2 expression in the Organ of Corti was observed, but only in the efferent innervation to inner and outer hair cells (Hossain *et al.*, 2005). The absence of evidence for expression of Nav1.8 and Nav1.9 isoforms in this study is in line with earlier observations that TTX completely abolished inward sodium current in isolated SGN (Santos-Sacchi, 1993). Nav1.4 and Nav1.5 expression in the SGN was not studied, as these isoforms are believed to be selectively expressed in skeletal and cardiac muscle respectively (Catterall, Goldin and Waxman, 2005).

Control results: The extensive analysis and comprehensive documentation of the expression of Nav subunits in the CNS and PNS neurons provided positive controls for RT-PCR primers used in the current study. CNS neurons have been shown to express Nav1.1, Nav1.2 (Westenbroek, Merrick and Catterall, 1989), Nav1.3 (Beckh *et al.*, 1989) and Nav1.6 (Tzoumaka *et al.*, 2000), and mRNA isolated from the cerebral cortex was used to test the specificity of the RT-PCR primers designed for these Nav subunits. Nav1.7 and Nav1.9 were also weakly identified by the RT-PCR primers. While these proteins have not been identified in the cerebral cortex, the mRNA has been detected in this tissue previously (Dib-Hajj *et al.*, 1998a, Belcher *et al.*, 1995). The mRNA isolated from the DRG was used as a positive control to validate the specificity of the RT-PCR primers designed to detect Nav1.7, Nav1.8 and Nav1.9, as the DRG has been previously reported to express these subunits (Sangameswaran *et al.*, 1997, Dib-Hajj *et al.*, 1998a, Dib-Hajj *et al.*, 1998b). The RT-PCR

primers also identified $\text{Na}_V1.1$ mRNA in the DRG tissues. This result has been reported previously (Beckh, 1990), but whilst the mRNA for $\text{Na}_V1.1$ has been identified in the DRG, the protein has not been detected in the peripheral nervous system (Gordon et al., 1987). Both these control tissues yielded results that confirmed that the designed primers were specific for their respective Na_V isoforms.

Antibody specificity is necessary for the reliable interpretation of immunohistochemical data. Sections of rat cochlea without primary antibodies did not display fluorescence specific to the binding of the secondary antibodies to their antigens. However, non-specific cytoplasmic yellow puncta were observed in the SG cell bodies. These results indicate that in the adult rat SGN there are other sources of endogenous fluorescence, possibly lipofuscin. This has also been reported in other adult neural ganglia, including the retina, vestibular and dorsal root ganglion and cerebellar Purkinje cells (Sans, Bartolami and Fraysse, 1996, Samorajski, Ordry and Rady-Reimer, 1968). Furthermore, we are confident that these puncta were due to non-specific fluorescence, since we used non-fluorescence DAB/HRP visualisation methods to validate the original results.

Characterising Na_V Isoforms in the Auditory pathway- Therapeutic Applications: The characterisation of Na_V isoforms in the periphery of the normal auditory pathway contribute to the development of therapeutics for treating conditions such as tinnitus. The clinical evidence that voltage-gated sodium blockers may have a role in ameliorating certain types of tinnitus is well documented because lignocaine, a use-dependent sodium channel blocker and local anaesthetic, is the gold standard tinnitolytic agent and is effective in around 50% of patients (Baguley et al., 2005). Following ototrauma, it is apparent that marked plastic changes occur in the auditory pathway some of which have parallels with sensory nerve

trauma (Moller, 1997, Kaltenbach et al., 1998). In models of neuropathic pain this is accompanied by changes in pattern and expression of Na_v subtype and profound changes in neuronal excitability (Devor, 2006, Novakovic et al., 1998, Black et al., 2004, Cummins and Waxman, 1997). Several Na_v channel subtypes including Na_v1.7 and Na_v1.8 have been implied to regulate spontaneous firing in damaged nociceptive afferents although there is much debate from studies using antisense knock-down methodologies and transgenic animals as to which are the most important (for reviews see Krafte and Bannion, 2008, Momin and Wood, 2008).

The use of appropriate models of ototrauma would establish whether similar or other changes in Na_v isoforms may underlie tinnitus. This would provide the basis for optimal matching of tinnitolytic therapeutics to their tinnitogenic target.

Experimental Methods

RT-PCR

Tissue extraction: Male Wistar rats (250-400g) were sacrificed using Euthanal® (IP) in accordance with UK Home Office Animals Act 1986 (schedule 1). The dorsal root ganglia (DRG) and cerebral cortex were rapidly excised and frozen over dry ice in a sterile RNase/DNase free tube. The temporal bones were removed and excess bone was removed to leave the bony inner ear segment that contained the cochlea. The otic capsule of the cochlea was removed to reveal the modiolus, which was then carefully removed from the remaining bone and frozen over dry ice as above.

mRNA isolation and cDNA synthesis: Two modioli- from the left and right ear of one animal- six DRGs and 35 mg of cerebral cortex were used per analysis. The tissues were homogenised separately in lysis buffer, by pestle and mortar, and centrifuged in a QIAshredder (QIAGEN, Crawley, UK). The total RNA was isolated from the homogenates using the RNeasy mini kit (QIAGEN). The RNA was eluted in RNase free water and the remaining DNA was removed by using DNase I (Invitrogen, Paisley, UK). The total RNA was reverse transcribed to complementary (c)DNA using SuperScript™ III Reverse Transcriptase (Invitrogen) The cDNA was stored at -80°C until use.

PCR reaction: The PCR reaction was performed using BIOTAQ DNA polymerase (Bioline, London, UK), custom designed sense and antisense primers at 0.5µM final concentration (Sigma Genosys, Cambridge, UK) and 1µl of cDNA using a T-Gradient Biometra thermocycler (Biotron, Göttingen, Germany). Thirty-five PCR cycles were performed using annealing temperatures between 54 and 59°C. The PCR products were separated by electrophoresis on a 1% agarose gel with ethidium bromide and a 100 base pair DNA ladder (Promega, Southampton, UK) at 110V. The gel was photographed under ultraviolet

illumination using a digital camera and Kodak digital science 1D software (Kodak). The mRNA isolation, cDNA synthesis and PCR reaction was repeated four times, using modiol from a fresh rat each time.

Primer design: Primer pairs specific to the Na_v alpha subunits and β -actin used are summarised in table 1. The primer pairs were designed to cross intron-exon boundaries as determined from the rat sequences. This would show any genomic DNA contamination of the PCR products as a higher base pair size band than the calculated band. The identity of PCR products was confirmed by sequencing.

Tissue preparation for immunohistochemistry

Dorsal root ganglion extraction: Male Wistar rats (250-300g) were sacrificed by stunning and cervical dislocation, in accordance with UK Home Office Animals Act 1986 (Schedule 1). The DRG were rapidly excised, embedded in Tissue-Tek OCT (Sakura Finetek, Bayer plc, Newbury, UK), frozen over dry ice/hexane and stored at -20°C . Frozen sections were cut (10 μm) from tissue blocks, mounted on PolysineTM slides (VWR International, Lutterworth, UK) and air-dried. Tissue sections were fixed in freshly prepared 2% paraformaldehyde solution for 10 minutes at room temperature and washed (1x15 mins) in phosphate buffered saline (PBS, pH 7.4).

Cochlea extraction: Male Wistar rats (250-300g) were sacrificed using Euthanal[®] (IP) in accordance with UK Home Office Animals Act 1986 (schedule 1). The circulatory system of the animal was flushed transcardially with warm PBS supplemented with heparin. For fixation the animal was perfused transcardially with warm 2% paraformaldehyde solution. The temporal bones were dissected and excess bone was removed to leave the bony inner ear segment that contained the cochlea. The cochleae were placed in 2% paraformaldehyde

solution for 30 minutes and demineralised in 8% EDTA until soft (4 to 10 days at 4°C). They were then placed in PBS with 30% sucrose overnight at 4°C and then were infiltrated with Tissue Tek OCT overnight at 4°C (Cho et al., 2004). The fixed cochleae were quickly frozen in Tissue Tek over dry ice/hexane and stored at -20°C. Frozen 10µm sections were cut from tissue blocks, mounted on PolysineTM slides and air dried. Tissue sections were washed (1x15 mins) in PBS.

Immunohistochemistry on frozen sections

Antibody information: Information about the primary and secondary antibodies used in this study is presented in table 2.

Fluorescent probe staining: For detection of sodium channel subtypes, the slides were incubated in the following: (1) Blocking solution of PBS containing 10% goat serum, supplemented with 0.5% Triton X-100 for 30 min at room temperature; (2) Sodium channel subtype specific primary polyclonal antibodies raised in rabbit (see table 2 for details) diluted in blocking solution and incubated overnight at 4°C; (3) Goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) for 2 hours at room temperature. Between each incubation step, the sections were thoroughly washed with PBS. Sections were mounted with Citifluor AF1 mountant (UKC Chem. Lab, Kent, UK).

Control experiments were carried out to determine the amount of non-specific binding by omitting the primary antiserum from the incubation media. Pre-incubation of primary antibody with the blocking peptide overnight reduced the labelling to background levels.

For identification of Type II SG neurons, experiments were carried out using an anti-peripherin monoclonal antibody MAB 1527 raised in mouse (Millipore, Watford, UK) as this protein is known to be selectively expressed in Type II SG neurons (Hafidi, 1998, Mou, Adamson and Davis, 1998, Reid, Flores-Otero and Davis, 2004). Immunostaining was visualised with goat anti-mouse secondary antibodies conjugated with Texas Red (Jackson ImmunoResearch).

The sections were viewed using a Nikon Labophot 2A microscope equipped for epifluorescence imaging and photography, with a monochromatic CCD camera, frame grabber card and software (Scion ImageTM). Photography of tissue sections was carried out with the appropriate filters for FITC or Texas Red.

Scion ImageTM (Scion Corporation, Frederick, Maryland USA) was used to measure the cell body area and average fluorescence of the neurons from the epifluorescent images. Only neurons that showed an unlabelled nucleus and an intact cell body were measured. The fluorescence scale ranged from: 0= black; 255= white. Unless otherwise stated, this scale is used throughout the results and discussion sections.

Non-fluorescence DAB/HRP staining: For non-fluorescence detection of sodium channel subtypes, the tissue sections were incubated sequentially in the following solutions: (1) 0.2% Hydrogen peroxide in methanol for 15 minutes; (2) 10% swine serum and 0.5% Triton X-100 in PBS for 30 minutes at room temperature; (3) Anti-rabbit polyclonal antibodies as described above, overnight at 4°C; (5) Swine anti-rabbit biotin conjugated IgG, diluted with 10% swine serum in PBS for 35 minutes at room temperature (DAKO, Ely, UK); (6) Streptavidin-Horseradish peroxidase (HRP) conjugate prediluted for 30 minutes at room

temperature (DAKO); (7) 3, 3'-diaminobenzidine solution (DAB substrate kit, DAKO) for 4 minutes at room temperature; (8) 70% IMS for 5 minutes followed by 100% IMS for 5 minutes and 100% xylene twice for 5 minutes. Between steps 1 and 8 the tissue sections were thoroughly washed with PBS. Sections were mounted using DPX (RA Lamb, Eastbourne, UK).

The slides were examined using a light microscope equipped with a digital camera (Moticam 2300, GT Vision, Haverhill, Suffolk, UK) and images were captured using Motic Images Plus 2.0

Statistical Analysis

Statistical analysis was carried out with data entered into a Microsoft Office Excel 2007 spreadsheet. Comparison of different data groups was performed using the two-tailed Students t-test. Results are expressed as means $\pm$ se and a $p=0.05$ significance level was adopted throughout.

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Subunit	Forward primer	Reverse primer	Predicted band (bp)	Annealing temperature (°C)
Na _v 1.1	GCGTGCTGCCGGGAAAACATAC	ACGTCCTTCGCTCGCCCTCTGA	576	59
Na _v 1.2	CGTCCGCAGAATCCAGAGA	CTTGAAGGGTCTTCCAACAAGTC	705	58
Na _v 1.3	AGGGAAGGATTGACTTGCC	TGGACCTCTCCTTAGAGTCCA	295	58
Na _v 1.6	AAGTGGACAGCCTACGGC	TTGTTGACAATATCGATTTTGAACCG	364	58
Na _v 1.7	GACGACAGCGGCACGACTAAT	AGCTGCGAAGATCCCTGTAAAGA	402	54
Na _v 1.8	GATCCGTGGAAGTGGCTGGA	GAGGAATGCCACGCAAAGGAATC	482	58
Na _v 1.9	CCCTGCTGCGCTCGGTGAAGAA	GACAAAGTAGATCCCAGAGG	395	58
β-actin	TACAACCTCCTTGCAGCTCC	GGATCTTCATGAGGTAGTCTGTC	630	59

Table 1. PCR Primers. Primers were either designed or modified from others previously published. Na_v1.2, Na_v1.3, Na_v1.6 (Wooltorton et al., 2007); Na_v1.8 (Dib-Hajj et al., 1998b); Na_v1.9 (Dib-Hajj et al., 1998a); β- actin (Raff et al., 1997).

Antibody	Host	Dilution	Supplier	Product code
Anti Na _v 1.1	Rabbit	1:200	Alomone Labs	ASC-001
Anti-Na _v 1.6	Rabbit	1:200	Alomone Labs	ASC-009
Anti-Na _v 1.7	Rabbit	1:200	Alomone Labs	ASC-008
Anti-Peripherin	Mouse	1:100	Millipore	MAB1527
Anti-rabbit/FITC	Goat	1:400	Jackson ImmunoResearch	111-095-144
Anti-mouse/Texas red	Goat	1:400	Jackson ImmunoResearch	115-075-146
Anti-rabbit/biotin	Swine	1:350	DAKO	E0353

Table 2. Immunohistochemistry antibodies. Details of the antibodies used in the study. Entries with / indicates a conjugation, i.e. anti-rabbit/biotin means anti-rabbit conjugated with biotin.

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Figure 1. Na_v mRNA channel expression in adult rat nervous tissues. PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide. (A) PCR products were detected for $\text{Nav}1.1$, 1.2 , 1.3 and 1.6 in the rat cerebral cortex, with weak bands for $\text{Na}_v1.7$ and $\text{Na}_v1.9$. (B) PCR products were detected for $\text{Nav}1.1$, 1.6 , 1.7 , 1.8 and 1.9 in the rat DRG. (C) PCR products were detected for $\text{Nav}1.1$, 1.6 and 1.7 in the rat SGN. All tissues were positive for β -actin. M lane, 100bp ladder with brightest band representing 500bp. Expected product sizes are given in methods section. PCR was performed either in the presence (+) or absence (-) of reverse transcription.

Figure 2. Distribution of Na_v1.7 in rat SGN. Micrographs showing Na_v1.7 labelling in the rat SGN using fluorescent (A,B) and non-fluorescent (D,E). Both techniques show positive labelling of the neurons although some appear to be less intensely stained (arrow) than others (arrowhead). (C) Micrograph showing rat SGN incubated without the primary antibody. (F) Phase contrast micrograph of HRP-DAB labelled SGN incubated without the primary antibody.

Scale bars= 30μm for all images.

Figure 3. Distribution of Na_v1.6 in rat SGN. Micrographs showing Na_v1.6 labelling in the rat SGN using fluorescent (A,B) and non-fluorescent (D,E) immunocytochemistry. Both techniques show positive labelling of the neurons although some appear to be less intensely stained (arrow) than others (arrowhead). (C) Micrograph showing rat SGN incubated without the primary antibody. (F) Phase contrast micrograph of HRP-DAB labelled SGN incubated without the primary antibody.

Scale bars= 30 μm for all images.

Figure 4. Distribution of Na_v1.1 in rat SGN. Micrographs showing Na_v1.1 labelling in the rat SGN using non-fluorescent (A,B) immunocytochemistry. (A,B) Faint labelling of the SGN cell bodies was observed (arrowhead) while the axons were more intensely labelled (arrow). (C) Phase contrast micrograph showing rat SGN incubated without the primary antibody.

Scale bars= 30μm for all images.

Figure 6. Dual distribution of Na_v1.6 and Peripherin in the rat SGN. Micrographs showing peripherin (A) and Na_v1.6 (B) labelling in the rat SGN. The neurons that are labelled for peripherin appear to have stained weakly for Na_v1.6 (arrows point to the position of peripherin positive neurons). Frequency histograms showing the distribution of spiral ganglion cell body areas (C) and normalised average fluorescent labelling for Na_v1.6 (D), with a scatter plot of neuronal cell body area versus fluorescent labelling for Na_v1.6 (E), showing that the peripherin positive neurons (clear bars/symbols) have smaller cell body area and label less intensely for Na_v1.6 than the peripherin negative neurons (filled bars/symbols). Scale bars=30μm. Graduated bar on graphs indicates fluorescence scale (0=black, 255=white).