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The TTX-resistant sodium channel Na$_v$1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons

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We have examined the distribution of the sensory neuron-specific Na$^+$ channel Na$_v$1.8 (SNS/PN3) in nociceptive and non-nociceptive dorsal root ganglion (DRG) neurons and whether its distribution is related to neuronal membrane properties. Na$_v$1.8-like immunoreactivity (Na$_v$1.8-LI) was examined with an affinity purified polyclonal antiserum (SNS11) in rat DRG neurons that were classified according to sensory receptive properties and by conduction velocity (CV) as C-, Aδ- or Aα/β. A significantly higher proportion of nociceptive than low threshold mechanoreceptive (LTM) neurons showed Na$_v$1.8-LI, and nociceptive neurons had significantly more intense immunoreactivity in their somata than LTM neurons. Results showed that 89, 93 and 60 % of C-, Aδ- and Aα/β-fibre nociceptive units respectively and 88 % of C-unresponsive units were positive. C-unresponsive units had electrical membrane properties similar to C-nociceptors and were considered to be nociceptive-type neurons. Weak positive Na$_v$1.8-LI was also present in some LTM units including a C LTM, all Aδ LTM units (D hair), about 10 % of cutaneous LTM Aα/β-units, but no muscle spindle afferent units. Na$_v$1.8-LI intensity was negatively correlated with soma size (all neurons) and with dorsal root CVs in A- but not C-fibre neurons. Na$_v$1.8-LI intensity was positively correlated with action potential (AP) duration (both rise and fall time) in A-fibre neurons and with AP rise time only in positive C-fibre neurons. It was also positively correlated with AP overshoot in positive neurons. Thus high levels of Na$_v$1.8 protein may contribute to the longer AP durations (especially in A-fibre neurons) and larger AP overshoots that are typical of nociceptors.

Somata of DRG neurons with nociceptive properties differ from those with low threshold mechanoreceptive (LTM) properties in their electrical membrane properties including broader/inflected action potentials (APs) (Koerber & Mendell, 1992; Ritter & Mendell, 1992; Djouhri *et al.* 1998; Gee *et al.* 1999) and larger AP overshoots (Djouhri & Lawson, 2001*a*). Ion channel subunits that are preferentially expressed in nociceptive neurons and may be responsible for these differences could be useful targets for novel therapeutic drugs for chronic pain treatment.

Initiation and propagation of APs depends on inward Na$^+$ currents. In adult DRG neurons, Na$^+$ currents include: TTX-sensitive fast-inactivating (TTX-S$_F$), TTX-resistant fast-inactivating (TTX-R$_F$), TTX-R slowly inactivating (TTX-R$_S$) and TTX-R persistent (TTX-R$_P$) currents (see Baker & Wood, 2001; Chung *et al.* 2003). Because TTX-S$_F$ currents are in all types of DRG neurons (Kostyuk *et al.* 1981; Caffrey *et al.* 1992; Roy & Narahashi, 1992), and TTX-R$_F$ is detectable in only ~3 % of small DRG neurons

of adult rat (Renganathan *et al.* 2002), neither is likely to be associated selectively with most nociceptive neurons. On the other hand, TTX-R$_S$ (Roy & Narahashi, 1992; Elliott & Elliott, 1993; Rush *et al.* 1998) and TTX-R$_P$ currents (Dib-Hajj *et al.* 2002; Tate *et al.* 1998) are primarily in smaller DRG neurons, making them candidates for expression in nociceptive neurons. Two α-subunits, Na$_v$1.8 and Na$_v$1.9, that have been cloned, are associated with these TTX-R currents. Na$_v$1.9 has a low (hyperpolarized) activation threshold and ultraslow inactivation, and gives rise to the TTX-R$_P$ current (Waxman, 1999; Cummins *et al.* 1999; Chung, 2003), while Na$_v$1.8, also known as SNS, PN3 or ScN10A (Akopian *et al.* 1996; Sangameswaran *et al.* 1996; Oaklander & Belzberg, 1997; Goldin *et al.* 2000), has a relatively depolarized (high) threshold of activation (−30 mV), and is thought to give rise to the TTX-R$_S$ inward Na$^+$ current and contribute to the inward current in TTX-R APs (Akopian *et al.* 1996; Renganathan *et al.* 2001; Chung *et al.* 2003). Although we have previously found that expression of Na$_v$1.9 was limited to nociceptive neurons in rat DRGs (Fang *et al.* 2002), the sensory

properties of DRG neurons expressing $Na_v1.8$ have not been studied.

The properties of $Na_v1.8$ and its expression (mRNA and protein) mainly in small–medium sized DRG neurons (Akopian *et al.* 1996; Sangameswaran *et al.* 1996; Tate *et al.* 1998; Novakovic *et al.* 1998; Amaya *et al.* 2000) are consistent with the known distribution of $TTX-R_S$ current and with its contribution to the high threshold TTX-R broad APs in nociceptive DRG neurons (Waddell & Lawson, 1990; Ritter & Mendell, 1992; Djouhri *et al.* 1998).

So far, the evidence linking this channel with nociceptors is indirect, being based on cell size and membrane properties mainly of isolated DRG neurons *in vitro*. However, the assumption that all small–medium sized neurons are nociceptive may be misleading, because there is overlap in cell size between DRG neurons with C-, Aδ- and Aα/β-fibres (Harper & Lawson, 1985) and ample evidence for nociceptive and LTM neurons with all these types of fibre (see review, Lawson, 2002). We have therefore examined directly whether $Na_v1.8$ is indeed expressed selectively or preferentially in nociceptive primary afferent neurons, and whether it is associated with electrophysiological properties in such neurons *in vivo*. Preliminary results of this study were reported in abstract form (Fang *et al.* 2001).

METHODS

Meticulous
Young female Wistar rats (weight 160–180 g) were anaesthetized with an initial dose of 65–75 mg kg^{-1} I.P. of sodium pentobarbitone (25 mg ml^{-1} in distilled water with 10 % ethanol). This anaesthetic dose produced deep anaesthesia with areflexia (i.e. total absence of limb withdrawal reflex). A tracheotomy was performed to allow artificial ventilation and end-tidal CO_2 monitoring. To enable regular intravenous (I.V.) injections of the additional doses of the anaesthetic (10 mg kg^{-1}) that were required to maintain this deep level of anaesthesia, the right carotid vein was cannulated. The left carotid artery was also cannulated to allow monitoring of blood pressure. The temperature in the paraffin pool near the DRG was maintained throughout at 28–32 °C. Experimental procedures complied throughout with UK Home Office Guidelines. Full details of the animal preparation were as reported previously in guinea-pig (Lawson *et al.* 1997; Djouhri & Lawson, 1999) and rat (Fang *et al.* 2002).

Just prior to electrophysiological recording all animals were given a muscle relaxant to improve stability during recording. This was pancuronium (0.5 mg kg^{-1} I.V.) accompanied always by an additional dose (10 mg kg^{-1}, I.V.) of the anaesthetic. The same doses of muscle relaxant and anaesthetic (always given together) were administered at approximately hourly intervals. These anaesthetic doses were the same as those that induced deep anaesthesia during the period (2–3 h) of animal preparation. Blood pressure measurements indicated that using these same doses of muscle relaxant and anaesthetic, the blood pressure remained stable showing no indication of any reduction in the depth of anaesthesia at any stage in the experiment.

Intracellular recordings
Intracellular recordings from DRG somata were made using glass microelectrodes filled with a fluorescent dye. The fluorescent dyes were Lucifer yellow CH (LY) (5 mg ml^{-1}) in 0.1 M LiCl, ethidium bromide (EB) (6 mM) in 1 M KCl or Cascade blue (CB) as a 3 % solution in 0.1 M LiCl. The microelectrode was advanced until a membrane potential was seen and an action potential (AP) could be evoked by dorsal root stimulation with single rectangular pulses (0.03 ms duration for A-fibre units or 0.3 ms for C-fibre units). The stimulus intensity was usually adjusted to twice threshold (for A-fibre units) and between one and two times threshold (for C-fibre units). The somatic APs were recorded online with a CED (Cambridge Electronic Design) 1401 plus interface and the SIGAV program from CED, and they were subsequently analysed offline with a script running in the Spike II program (CED). For each unit, a number of AP variables were measured as described previously (see Djouhri & Lawson, 1999). These included the AP duration at base (AP duration), AP rise time (time from inception of the AP to the peak) and AP fall time (time from the peak to the point where the potential crosses or reaches the resting membrane potential (V_m)). AP overshoots were examined as previously described (Djouhri & Lawson, 2001*a*); however, unlike that study, we were limited to cells recorded with dye-filled electrodes, some of which had high resistances (> 400 MΩ). While this may have affected the height of some APs, there was no correlation in the study of Djouhri & Lawson (2001*a*) between AP height and electrode resistance. Even if there was an effect, this would introduce non-systematic scatter into the data rather than affecting patterns related to $Na_v1.8$-like immunoreactivity ($Na_v1.8$-LI) intensity.

The conduction velocity (CV) of each unit was estimated from the latency to onset of the dorsal root evoked somatic AP and the conduction distance. The latter was measured at the end of the experiment from the cathode of the stimulating electrode pair on the dorsal root to the approximate (±0.25 mm) location of the injected neuron in the DRG and was between 4.5 and 13 mm. Utilization time was not taken into account. Neurons were classified as C-, Aδ- and Aα/β-units with C cells conducting in the dorsal root at < 0.8 m s^{-1}, C/Aδ at 0.8–1.4 m s^{-1}, Aδ at 1.4–6.5 m s^{-1} and Aα/β at > 6.5 m s^{-1}. This classification was based on compound AP recordings from L5 and L6 dorsal roots in rats of the same sex and weight, and paraffin pool temperatures as in this experimental series (see Fang *et al.* 2002) using methods as described by Djouhri & Lawson (2001*b*).

Sensory receptive properties
The sensory receptive properties of DRG neurons were identified and classified as described previously (Lawson *et al.* 1997; Djouhri *et al.* 1998). Briefly, A-fibre low threshold mechanoreceptive (LTM) units were those that responded to non-noxious mechanical stimuli including light brush of the limb fur, light skin contact and light pressure with blunt objects, light tap, tuning forks vibrating at 100 or 250 Hz and light pressure with calibrated von Frey hairs. Most Aα/β LTM units in this study were cutaneous, with superficial or dermal receptive fields (see Lawson *et al.* 1997), but a group of non-cutaneous Aα/β-fibre LTM units that responded to light pressure against muscle tissue and that followed vibration of 100 or 250 Hz applied with a tuning fork and which often showed ongoing discharges were classified as muscle spindle (MS) afferent units. This group included group I and II muscle afferents and probably also Golgi tendon organ afferents. Aδ LTM down hair (D hair) units were those with Aδ-fibres that were extremely sensitive to slow movement of hairs, to stretch and

to cooling stimuli. Cooling of the skin was usually achieved with a very brief spray with ethyl chloride. C LTM units (also known as C mechanoreceptors) were those units that responded preferentially to very gentle contact moving across the skin at < 1 mm s^{-1} and sometimes to cooling as previously reported in several species (e.g. Light & Perl, 1993; Djouhri *et al.* 1998).

Units not responding to the above low intensity stimuli but responding either to noxious mechanical stimuli applied with a needle, fine forceps or coarse toothed forceps, or to both noxious mechanical and noxious heat (hot water at > 50 °C) stimuli were classified as nociceptive neurons. The approximate depth of the receptive field was determined from the minimum amount (depth) of tissue that was stimulated in order to evoke a response, and thus response to a needle prick or stimulation of very superficial tissue with the finest (no. 5) forceps was interpreted as a superficial cutaneous (epidermal or superficial dermal) receptive field. If squeeze of tissue including the dermis was required, this was interpreted as a deep cutaneous (dermal) field, and if squeeze of deeper tissues was required the field depth was designated as 'deep'. A-fibre nociceptive units in this study included: (a) Aδ- and Aα/β-fibre high threshold mechano-receptive (A HTM) units responding to noxious mechanical stimuli, (b) A mechano-cooling (A MC) responding to both noxious mechanical stimuli and cooling. No A-mechano-heat units were encountered in these experiments, although we have found small numbers of these in other experiments in guinea-pig (Lawson *et al.* 1997) and rat (L. Djouhri, X. Fang & S. N. Lawson, unpublished observations). C-fibre nociceptive units in this study include units that responded vigorously to both noxious heat and noxious mechanical stimuli; these we called C polymodal units (C PM) if their receptive fields were in the superficial cutaneous tissue or mechano-heat (C MH) units if their receptive fields were in the deep cutaneous tissues. They also include cutaneous C HTM units that required strong mechanical stimulation but lacked prompt responses to noxious heat and C HTM units with deep receptive fields that responded to strong mechanical stimulation but were not tested with thermal noxious stimuli. Specific C-fibre heat and cooling units were not found in this study, although we found C cooling units in similar studies in guinea-pig (Lawson *et al.* 1997) and rat (L. Djouhri, X. Fang & S. N. Lawson, unpublished observations).

Units that showed no response to the above non-noxious and noxious mechanical and thermal stimuli are called unresponsive. These unresponsive neurons have been described in several species (Meyer *et al.* 1991; Handwerker *et al.* 1991; Schmidt *et al.* 1995; Gee *et al.* 1996; Djouhri *et al.* 1998). They may be nociceptive units with extremely high thresholds, so-called 'silent nociceptors', or units for which the appropriate stimuli (e.g. inflammatory mediators) were not applied or which had inaccessible receptive fields. We found that C-fibre unresponsive units had long AP and AHP durations and large AP overshoots (values not shown) typical of nociceptive C-fibre units both in this study and in the guinea-pig (see Djouhri *et al.* (1998) and Djouhri & Lawson (2001*a*)). The C-unresponsive units were therefore called 'nociceptive-type' neurons in contrast to C LTM units (Djouhri *et al.* 1998) that had much lower values for AP and AHP durations and AP overshoots. The term 'nociceptive-type' is therefore used to encompass C- and A-fibre nociceptive neurons and C-unresponsive neurons. In contrast A-fibre unresponsive units were not included as nociceptor-type units for the following reasons. There were only two Aδ-fibre unresponsive units, again

with properties similar to nociceptive units, but too few to form a separate group. Many Aα/β-fibre unresponsive neurons had AP and AHP durations closer to those of the Aα/β LTM units and may therefore have included LTM units with inaccessible receptive fields. Because of small numbers and their mixed properties, A-fibre unresponsive units were not included as a separate group in the analyses to follow.

Fluorescent dye injection

Following characterization of each cell, a dye (LY, EB or CB) was ejected into the soma electrophoretically by rectangular current pulses. These were usually 1 nA, maximum 1.3 nA, for 500 ms at 1 Hz for periods of up to 10–15 min for A-fibre neurons and 6–10 min for C-fibre neurons. Membrane potential and receptive field response were monitored throughout this time. The currents passed were negative for LY and CB and positive for EB. L5 DRGs were about 2 mm long. The usual procedure was to label three neurons in L5 DRGs with LY (two at opposite ends of the ganglion and one in the middle), two further neurons with EB, between the first and second, and between the second and third LY-labelled neurons, respectively. In addition, sometimes neurons were labelled with CB at locations lateral to the tracks made with LY electrodes. The problems associated with identifying the dye-injected cells have been discussed (Lawson *et al.* 1997) and all precautions outlined in that report were taken and are now routine procedure.

Under deep anaesthesia, each animal was terminally perfused through the heart with 0.9 % saline followed by Zamboni's fixative (Stefanini *et al.* 1967) at the end of the experiment. The DRGs were dissected out, post-fixed for about 1 h and left overnight in 30 % sucrose in 0.1 M phosphate buffer at 4 °C. Then serial 7 μm cryostat sections were cut and mounted on 20 slides so that on each slide there was a series made up of every 20th section. Each section was examined with fluorescence microscopy and fluorescently labelled neurons were recorded with camera lucida drawings and images captured with a high resolution CCD camera (Optronix Dei-470).

Polyclonal antibody production and Western blotting

Antisera were raised following methods outlined in Harlow & Lane (1988) by immunizing rabbits to an epitope in Na$_v$1.8 (residues 1892–1956), which has no homology to other known Na$^+$ channels.

Antibodies were affinity purified on peptide columns before use. Western blot tests were carried out as follows. Tissues from 3-week-old Sprague-Dawley rats were homogenized in 10 mM phosphate buffer (pH 7.4) and centrifuged at 2000 *g* for 10 min. The supernatant was centrifuged again at 200 000 *g* for 60 min. The pellet was solubilized in 10 mM phosphate buffer containing 2 % Triton X-100 and 4 % SDS. This plasma membrane fraction was electrophoresed on SDS–5 % PAGE (0.2 mg lane^{-1}) and transferred to a Hybond ECL nitrocellulose filter (Amersham Pharmacia Biotech) using a wet electrophoretic transfer system. The membrane was blocked for 1 h at room temperature in 5 % non-fat dry milk in PBS-T (0.1 % Tween-20 in PBS, pH 7.4). The blot was probed with polyclonal antibody to Na$_v$1.8 (called SNS11) at 1:10 000 dilution for 1 h at room temperature and visualized with horseradish peroxidase-conjugated sheep anti-rabbit IgG (Amersham Pharmacia Biotech) in 1:2000 dilution using the ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech) and the blot was exposed to BioMax film (Kodak).

Anti-Na$_V$1.8 antibody SNS11 specifically recognizes Na$_V$1.8

Western blot analysis of the subtype specificity of anti-Na$_v$1.8 polyclonal antibody SNS11 showed a single band of strong immunoreactivity at approximately 240 kDa in the membrane fraction of rat DRG (Fig. 1*A*) which is consistent with the result obtained with the anti-Na$_v$1.8 monoclonal antibody 9D11C8 (Okuse *et al.* 1997). Membrane fractions of rat whole brain, cerebellum, skeletal muscle, heart and superior cervical ganglia (SCG), which are known to express other types of voltage-gated Na$^+$ channel such as Na$_v$1.1, 1.2, 1.4, 1.5, 1.6 and 1.7, and membrane fractions from the liver and the kidney all showed no detectable immunoreactivity. The membrane extract from CHO-SNS22, a Chinese hamster ovary cell line stably transfected with rat Na$_v$1.8 cDNA (Okuse *et al.* 2002), showed a strong band of immunoreactivity at a similar size to the DRG band, while non-transfected CHO cells did not show any bands. The finding of immunoreactive bands only in the membrane extracts from rat DRG and CHO-SNS22 cells suggest that the anti-Na$_v$1.8 polyclonal antibody SNS11 specifically recognizes Na$_v$1.8.

Immunocytochemistry

Details of the methods of assessing relative intensity of an immunocytochemical reaction product in DRG neurons have been reported previously (Lawson *et al.* 1997, 2002; Fang *et al.* 2002). Briefly, after block of endogenous peroxidase with H$_2$O$_2$, and blocking of endogenous biotin-like activity using an Avidin-Biotin kit (Vector laboratories, Burlingame, CA, USA), sections were first incubated for 2 days at 4°C in primary antibody (SNS11) against the Na$_v$1.8α-subunit (1.7×10^{-3} μg ml^{-1}) in 0.3% Triton X-100 in Tris buffer with 1% normal goat serum and they were then incubated for 30 min at room temperature with biotinylated secondary antibody (anti-rabbit Ig, 1:200) (Vector Laboratories). Diaminobenzidine (DAB) was used to form a coloured reaction product. The sections were dehydrated and coverslips were placed on the slides. Non-specific staining with secondary antibody was not found in the absence of primary antibody.

Cell size

The cross-sectional area of the largest section through each dye-injected neuron was used an indication of its size. In addition, for comparison with the dye-injected experimental neurons a general size distribution of DRG neurons was generated. For this, cross-sectional areas were measured of all neuronal profiles containing a nucleus in transverse DRG sections taken at 420 μm intervals through one L5 DRG from each of three female rats with weights near the mid-range used in these experiments.

Assessment of relative intensity of Na$_v$1.8-LI

For each dye-injected neuron, the intensity of Na$_v$1.8-LI was scored subjectively from 0 to 5, and then, using image analysis techniques, the intensity relative to other cells in that section was calculated as a percentage of maximum staining intensity seen in that section, as previously described (Fang *et al.* 2002). There was a highly significant linear correlation between objective scores of relative intensity and subjective scores of Na$_v$1.8-LI with $r^2 = 0.93$ ($P < 0.0001$), suggesting a close agreement between subjective and objective scores (Fig. 1*B*). All neurons categorized subjectively as positive ($\geqslant 1$) had a relative intensity $\geqslant 20\%$ and vice versa. Accordingly, a relative intensity of 20% was used as the borderline between negative and positive neurons in this study, although we cannot exclude the possibility that some cells with absorbance values of $< 20\%$ may express low levels of the protein.

As a comparison the relative intensities of neurons from three L5 DRGs were calculated as follows: the minimum (0%) was the average overall pixel intensity in the cytoplasm for the 10 least intensely labelled neurons and the maximum (100%) was the average for the 10 most intensely labelled cells; each cell was given a percentage value on a linear scale scaled between these two values (see Fang *et al.* 2002).

Acceptance criteria

Data were included only if the temperature at the DRG was 28–32°C. For the analysis of the AP characteristics, data were included only if the somatic AP was overshooting, and neurons
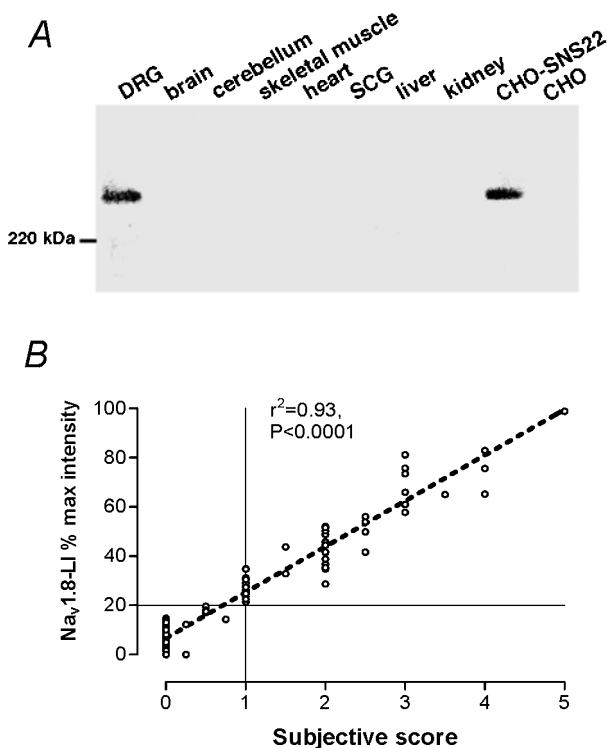

Figure 1. Western blot analysis and relationship of subjective score to relative intensity of Na$_v$1.8-LI

A, Western blot analysis. A single band of strong immunoreactivity at approximately 240 kDa in the membrane fraction of rat DRG is shown. Note that membrane fractions of rat whole brain, cerebellum, skeletal muscle, heart, superior cervical ganglia, liver and kidney showed no detectable immunoreactivity. Membrane extract from CHO-SNS22 (a cell line stably transfected with rat Na$_V$1.8 cDNA) showed strong immunoreactivity at the same size with DRG. Non-transfected CHO cells did not show any bands. *B*, relationship of subjective score to relative intensity of Na$_v$1.8-LI. The *x*-axis shows the subjective score (0 for the most negative, 1 for just positive and 5 for the most intense Na$_v$1.8-LI). The *y*-axis shows the percentage of relative intensity of Na$_v$1.8-LI from 0 to 100%, ranging from the most negative (0%) to the most intense Na$_v$1.8-LI (100%).

had membrane potentials more negative than −50 mV; the exception was a single C LTM unit with membrane potential of −48 mV. Units with a marked inflection on the AP rising phase were excluded from the AP analysis ($n = 2$, both C-fibre units) as these were atypical.

RESULTS

A total of 104 neurons with identified sensory receptive properties, labelled with a fluorescent dye and successfully recovered from histology, were examined for Na$_v$1.8-LI. Of these neurons, 47 were labelled with Lucifer yellow (LY), 45 with ethidium bromide (EB) and 12 with Cascade blue (CB). The DRGs were L5 for 97 neurons, L4 for one neuron and L6 for six neurons. Of the total sample, 38 were nociceptive units (9 C-, 1 C/Aδ-, 13 Aδ- and 15 Aα/β-fibres), 18 were unresponsive (8 C-, 1 C/Aδ-, 1 Aδ- and 8 Aα/β-fibres) and 48 were LTMs (1 C-, 4 Aδ- and 43 Aα/β-fibres). The Aα/β LTM units included 16 field or guard hair (F/G) neurons, 12 rapidly adapting (RA), 3 slowly adapting (SA) and 12 muscle spindle (MS) afferent neurons. Unless otherwise stated the C/Aδ-neurons are included with the Aδ-neurons in the following analyses.
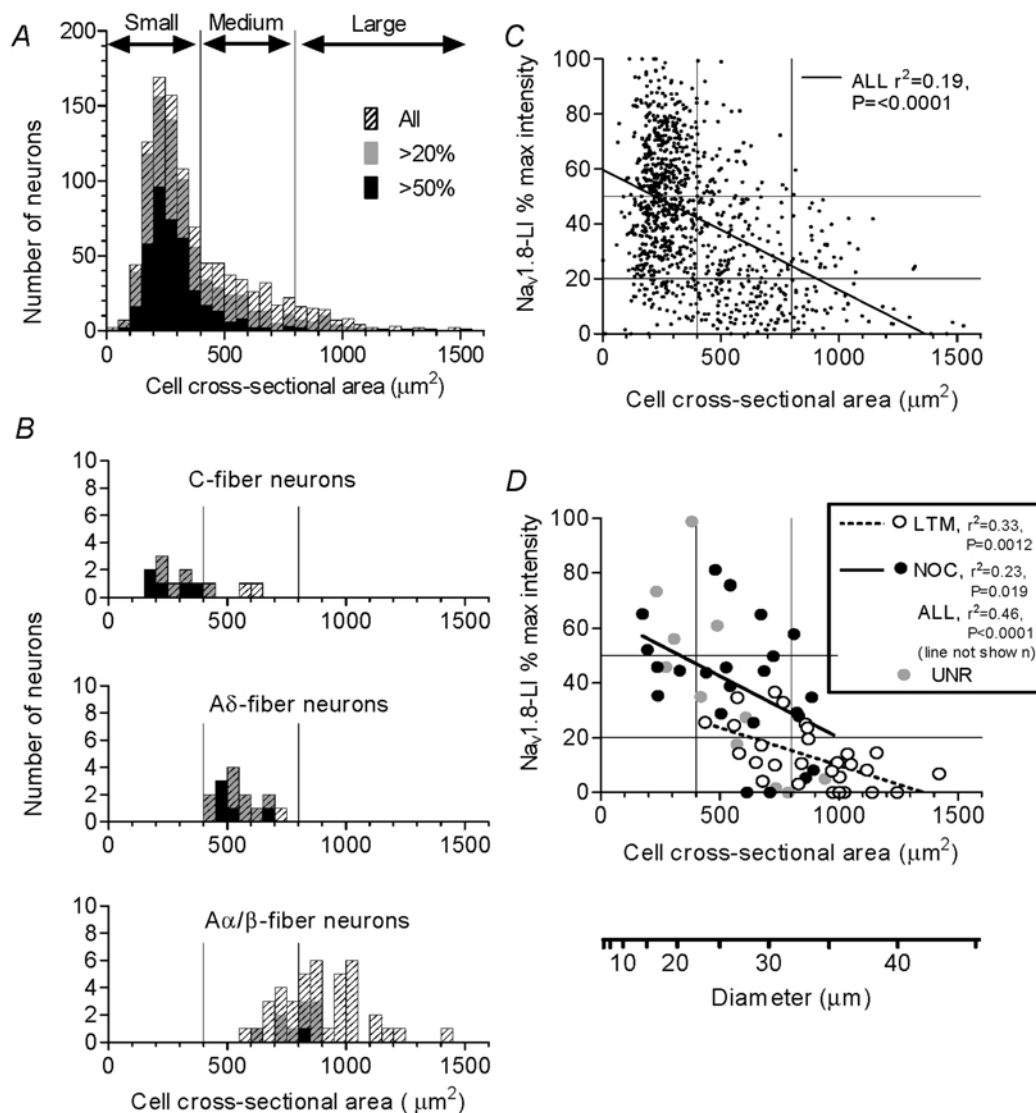

Figure 2. Cell area and Na$_v$1.8-LI relative intensity

The cross-sectional areas (*x*-axis) through each neuronal profile containing a nucleus (*A*) and through the largest section of each identified dye-injected neuron (*B*) are plotted against the relative intensity (percentage maximum intensity). In both cases, all neurons are shown as hatched histograms. Superimposed in grey are neurons with Na$_v$1.8-LI relative intensity > 20 % and superimposed in black are those with > 50 % maximum intensity. *B*, histograms for neurons with dorsal root C-, Aδ- and Aα/β-fibres are displayed separately. *C* and *D*, relative intensity is plotted against cell size. The lines (*y*-axis) show 20 and 50 % relative intensity, and those on the *x*-axis show the borderlines between small, medium and large neurons. *C* and *D*, regression lines, r^2 and *P* values are given where a significant correlation exists ($P < 0.05$). LTM, low threshold mechanoreceptors; NOC, nociceptors; UNR, unresponsive units.
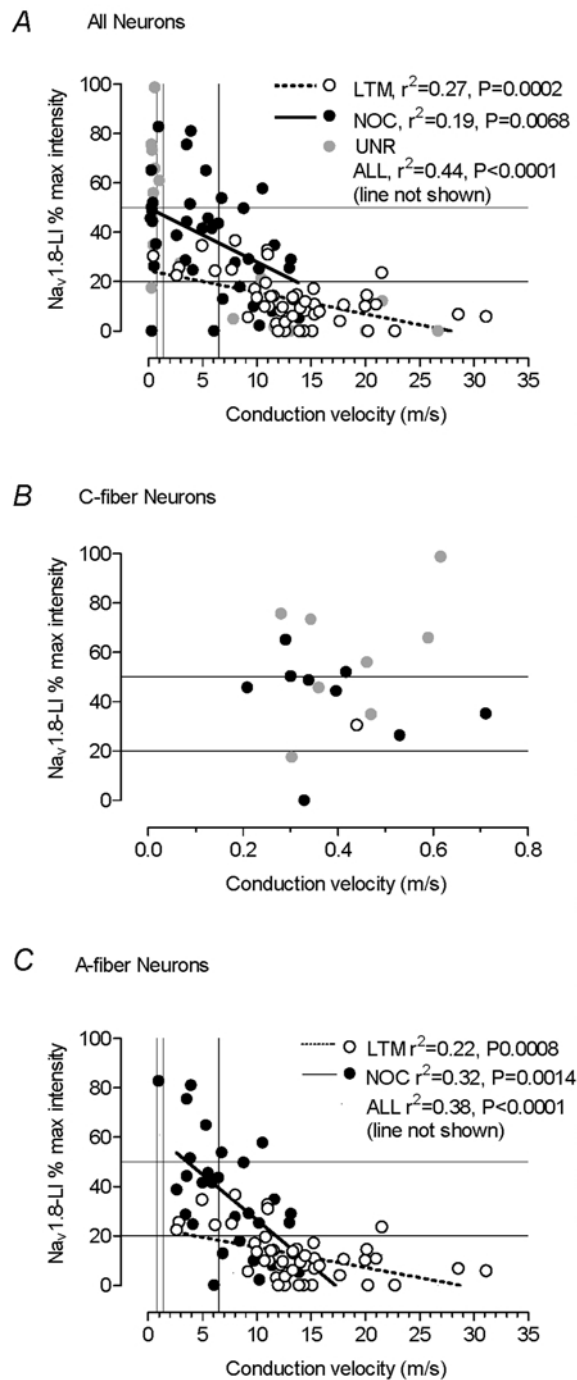
Figure 3. $Na_v1.8$-LI intensity and conduction velocity

$Na_v1.8$-LI intensity plotted against dorsal root CVs for all DRG neurons examined (*A*) and for all A-fibre neurons (*B*). The *P* and r^2 values are given only if the correlation is significant ($P < 0.05$). Both the borderline between $Na_v1.8$-LI-positive and -negative neurons (20 % maximum intensity) and 50 % maximum intensity are shown by lines on the *y*-axis. The lines from the *x*-axes show the C (0.8 m s^{-1}), C/Aδ (1.4 m s^{-1}) and the Aδ/Aα/β (6.5 m s^{-1}) borderlines. NOC, nociceptive neurons; LTM, low threshold mechanoreceptive neurons; UNR, unresponsive neurons.

Cell size

The relationship between cell size and $Na_v1.8$-LI is shown in Fig. 2, in *A* and *C* for all cells in the DRG and in *B* and *D* for the 72 dye-injected neurons for which the largest section was available for measurement. The overall size distribution in Fig. 2*A* was used to divide neurons into small ($< 400~\mu m^2$, 23 μm diameter, within the small cell peak), medium-sized (400–$800~\mu m^2$, 23–$32~\mu$m diameter) and large (above 800 μm^2 or 32 μm) including only the right hand side (larger cells) of the 'light cell' distribution (Lawson *et al.* 1984). Although many groups use 30 μm diameter ($\sim 700~\mu m^2$ cross-sectional area) as the upper limit for 'small' DRG neurons, for the animals and tissue processing in the present study, this range would include most medium-sized neurons and is therefore not appropriate.

Most small neurons, both in the whole DRG (Fig. 2*A*) and in the group of dye-injected neurons (Fig. 2*B*), were $Na_v1.8$-positive and the strongest immunoreactivity was in the smallest neurons in both cases (Fig. 2*C* and *D*). In addition, a few medium-sized neurons showed strong immunoreactivity, and even some large neurons also showed clear immunoreactivity (Fig. 2*A*). In Fig. 2*B*, C-fibre neurons were mainly small and most were positive, all Aδ-fibre neurons were medium-sized and most were positive, while Aα/β-fibre neurons were medium and large, including both medium and large positive neurons, but none of the very largest neurons ($> 900~\mu m^2$) were positive. Lower percentages of Aα/β-fibre neurons compared with Aδ- or C-fibre neurons were positive. These percentages were, respectively, for Aα/β-, Aδ- and C-fibre neurons: for positive neurons (> 20 %) 25, 93 and 83 %, and for strongly positive neurons (> 50 %) 3, 33 and 42 %. Note that the percentage classed as positive depends on the borderline chosen (here 20 or 50 % maximum intensity) between positive and negative. Figure 2*C* shows a highly significant negative correlation between cell size and relative intensity, albeit with a high variability of intensity at any given cell size, leading to a low r^2 value. There is a negative correlation between cell size and intensity for all cells and for nociceptive and LTM neurons separately (Fig. 2*D*).

The similarity in pattern (Fig. 2*D*) of the relationship between cell size and intensity of immunoreactivity in non-experimental and dye-injected neurons indicates no detectable effect of recording and dye injection on the level of immunoreactivity.

Conduction velocity

The relative intensity of $Na_v1.8$-LI was negatively correlated with dorsal root CV in all neurons ($n = 104$), as well as in nociceptive and LTM neurons separately, with the majority of strongly (> 50 % intensity) labelled units

being in the C- and Aδ-fibre range (Fig. 3*A*). Similar correlations were seen for A-fibre units alone (Fig. 3*C*) but there was no correlation for C-fibre units alone (Fig. 3*B*), in direct contrast to the findings for Na$_v$1.9 (Fang *et al.* 2002). There was a highly significant difference between the elevations but not the slopes (Prism 3) of regression lines for nociceptive and LTM data shown in Fig. 3*A* (all units) and *C* (A-fibre units) ($P < 0.001$ in both cases). Only one of the fastest ($> 15\text{m s}^{-1}$) conducting units was positive and that was only weakly positive (Fig. 3*A*).

Nociceptive *versus* LTM properties

Nociceptive neurons grouped together regardless of CV had a greater (highly significant) median relative intensity than similarly grouped LTM neurons; none of the LTM units had a relative intensity $> 40\%$, whereas half the nociceptive units had values of $> 40\%$ (Fig. 4*A*). A significantly greater proportion of nociceptive than LTM neurons was positive ($\geqslant 20\%$ relative intensity) (Fig. 4*B*). These differences are probably underestimated due to the under-representation in the identified sample of the C-fibre neurons many of which are nociceptive and show relatively strong immunoreactivity.

Nociceptive and non-nociceptive neurons with C- and A-fibres

Figure 4*C* shows the relative intensity of Na$_v$1.8-LI in different subgroups of identified neurons. The most intensely labelled neurons were nociceptive (C, Aδ and Aα/β) and C-unresponsive neurons; only these neurons had relative intensities of $> 40\%$. Examples of Na$_v$1.8-LI in C- and A-fibre neurons with identified sensory properties are shown in Fig. 5.

C-fibre units. Most (8/9, 89 %) C-fibre nociceptive units were clearly positive. Positive units included six HTMs, two HTM-cooling (MC) units and one unit responsive to noxious heat (C PM). The only Na$_v$1.8-LI negative C-fibre nociceptive neuron was a C HTM unit. Interestingly, most (7/8, 87 %) C-fibre unresponsive units were clearly positive with relatively high intensity of labelling (for example see Fig. 5). The only C-fibre LTM unit was weakly positive and was among the few C-fibre units with Na$_v$1.8-LI intensity of $< 40\%$.

Aδ-fibre units. Almost all Aδ fibre nociceptive units (13/14, 93 %) were clearly positive (including the only C/Aδ-fibre nociceptive unit which was strongly positive at

Figure 4. Na$_v$1.8-LI intensity in different neuronal types

A, scattergraph of the relative intensities of nociceptive and LTM units in all CV groups together shows a much greater median relative intensity in nociceptors (Mann-Whitney test, $P < 0.0001$). *B*, the proportions of positive ($< 20\%$, black) and negative (white) nociceptive and LTM units from all CV groups together are compared (χ^2 test, $P < 0001$). *C*, neurons are subdivided by sensory modality within C, Aδ and Aα/β CV groups. The borderline between negative ($< 20\%$) and positive ($\geqslant 20\%$) neurons is indicated by the line at 20 % relative intensity. Abbreviations: NOCI, nociceptive neurons; LTM, low threshold mechanoreceptive neurons; UNR, unresponsive neurons; F/G, field or guard hair LTM neurons; RA, rapidly adapting LTM neurons; SA, slowly adapting LTM neurons; MS, muscle spindle neurons; +ve, Na$_v$1.8-LI-positive ($\geqslant 20\%$) neurons; −ve, Na$_v$1.8-LI-negative ($< 20\%$) neurons.
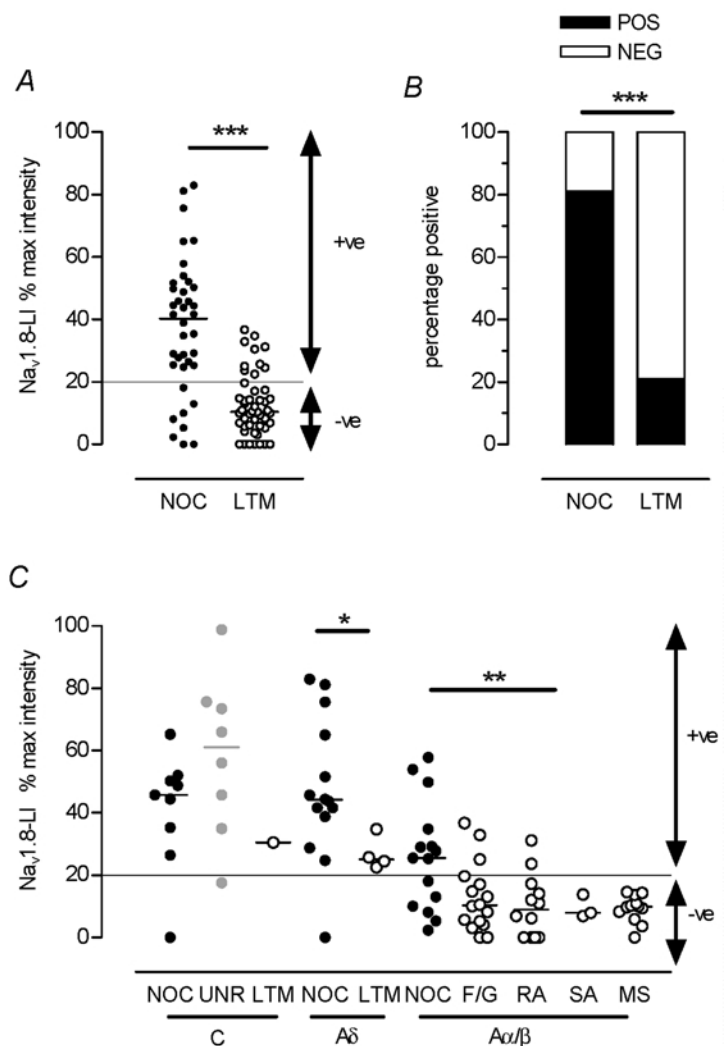
Table 1. Na_v1.8-LI relative intensity and action potential duration

Intensity vs. AP	All			All C positive			All A positive			All A			A LTM			A Nociceptive		
	P	r^2	n	P	r^2	n	P	r^2	n	P	r^2	n	P	r^2	n	P	r^2	n
Duration at base	****	0.4	41	ns	—	8	*	0.49	12	****	0.61	32	ns	—	21	**	0.58	11
	Slope +7.3 ± 1.4			—			Slope +23 ± 7.4			Slope +26.6 ± 3.8			—			Slope +35.6 ± 10.1		
Rise time	****	0.51	41	**	0.85	8	*	0.42	12	****	0.64	32	**	0.37	21	*	0.42	11
	Slope +24.5 ± 3.8			Slope +16.3 ± 2.8			Slope +44.8 ± 16.5			Slope +60.8 ± 8.4			Slope +44.7 ± 13.3			Slope +59.4 ± 23		
Fall time	***	0.31	41	ns	—	8	*	0.44	12	****	0.51	32	ns	—	21	**	0.59	11
	Slope +8.9 ± 3.1			—			Slope +40 ± 13			Slope +40.6 ± 7.2			—			Slope +65.3 ± 18		

Linear regression analysis was carried out on the groups indicated to examine the correlation of Na_v 1.8-like immunoreactivity (Na_v1.8-LI) relative intensity with AP duration at base, AP rise time and AP fall time. A-fibre unresponsive units were excluded. All neurons had membrane potentials (E_m) equal to or more negative than −50mV, except for the only C LTM (−48mV). All correlations were positive. In each case where $P < 0.05$, on the first line the P, r^2 and n values are given, and on the second line the slope is shown. ns, Not significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Plots of these correlations are shown in Fig. 6.

83 %), see Fig. 4*C*, Table 1. These positive neurons included 11 HTM and two mechano-cooling (MC) units; the negative unit was an HTM with a deep receptive field. A positive HTM unit is shown in Fig. 5. The only two unresponsive Aδ-fibre units were positive (one with a CV of 3 m s^{-1} and intensity of 27 % and the other 1 m s^{-1} and intensity of 61 %). Strong Na_v1.8-LI ($\geqslant$ 50 %) was only in nociceptive neurons, although weak but clear positive immunoreactivity was also seen in all four Aδ LTM (D hair) units (23–35 %), for example see Fig. 5.

Aα/β-fibre units. Only 21 % (14/66) of the total Aα/β-fibre neurons were Na_v1.8 positive (Fig. 4*C*). Of those that were positive, most (9/14, 64 %) were nociceptive. Of the nociceptive neurons, 60 % (9/15) were positive; an example of a moderately labelled Aα/β-fibre nociceptive unit can be seen in Fig. 5. The only neurons with Na_v1.8 intensity of > 40 % were nociceptive. All unresponsive Aα/β-fibre units ($n = 8$) were negative (not shown). Since most of these unresponsive Aα/β-fibre units had electrophysiological properties typical of LTM units (short AP and AHP durations), they were likely to include LTMs with inaccessible receptive fields; they were therefore not included in subsequent analyses. About 10 % (5/43) of LTM Aα/β-fibre units exhibited weak positive Na_v1.8-LI. These included three (F/G) field or guard hair (intensity 25–37 %) and two rapidly adapting (RA) neurons (intensities 24–31 %). An example of a borderline Aα/β LTM (G hair/field) unit can be seen in Fig. 5. None of the MS and SA neurons was positive for Na_v1.8-LI.

Na_v1.8-LI and action potential duration

In some DRG neurons TTX-R currents contribute to the AP inward current and may therefore influence the AP configuration. Correlation between Na_v1.8-LI intensity and AP duration at base, AP rise time and AP fall time were therefore examined. Only neurons with membrane potentials equal to or more negative than −50 mV plus a C LTM with a membrane potential of −48 mV were

included. Similar patterns and correlations were seen when neurons with membrane potentials between −40 and −50 mV were also included (not shown). All units included had overshooting APs. Positive correlations for all A-fibre neurons and for A-fibre nociceptive neurons were very clear, especially with AP fall time; however, for A-fibre LTMs a significant correlation was only found for AP rise time (Fig. 6). C-fibre neurons showed no correlation between Na_v1.8-LI intensity and AP duration at base (Fig. 6*A*) or AP rise time in contrast to A-fibre neurons. There was, however, a correlation with AP rise time both for C-nociceptors (Fig. 6) and for all positive C-fibre units (nociceptors, unresponsive and an LTM) (Fig. 6 and Table 1).

Membrane potential. There was no correlation of Na_v1.8-LI with membrane potential for A-fibre neurons, for all nociceptive or for all LTM neurons. However, there was a significant negative correlation between membrane potential and Na_v1.8-LI in C-fibre nociceptors but not C-fibre unresponsive units (Fig. 7) such that more intense immunoreactivity was seen in C-nociceptors with more negative membrane potentials. We have no explanation for this unexpected finding, since no difference was seen in membrane potentials in Na_v1.8−/− DRG neurons compared with wild-type (Renganathan *et al.* 2001). While it is possible that the expression of Na_v1.8 might be influenced by membrane potential in C-fibre nociceptors, we know of no other evidence for this. In addition, since real membrane potential is notoriously difficult to assess accurately, and may vary during recording, it may be misleading to emphasise this observation. There was no significant correlation between membrane potential and AP rise time or AP fall time for C-fibre nociceptive neurons, making it unlikely that the correlation of Na_v1.8-LI with membrane potential led indirectly to the correlation in C-fibre nociceptors between Na_v1.8 and AP fall time.
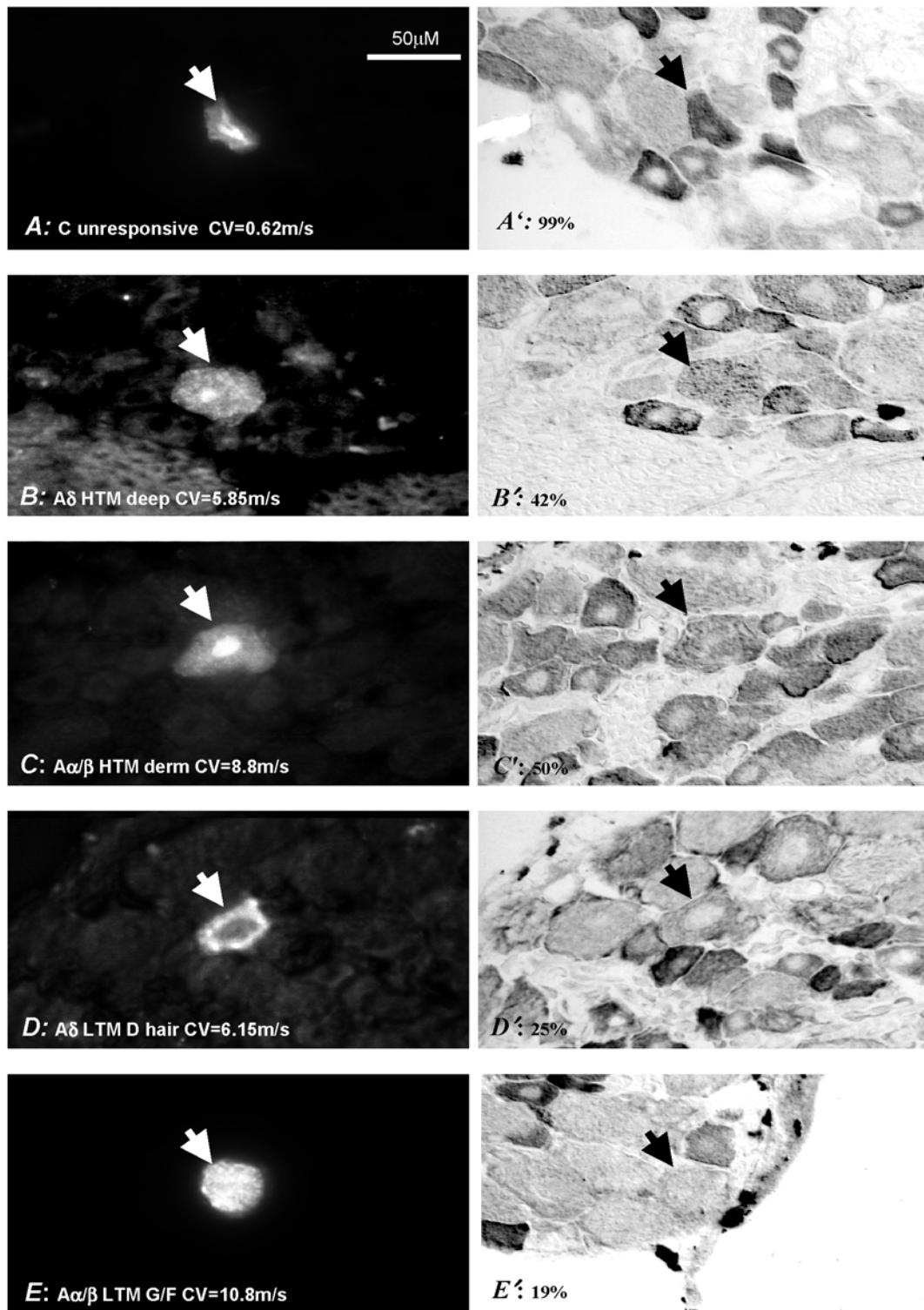
Figure 5. Examples of Na$_v$1.8-LI in identified neurons

Shown on the left are 5 DRG neurons injected with a fluorescent dye (marked with arrowheads) and on the right are the same neurons stained for Na$_v$1.8-LI. Their sensory properties, dorsal root CV (bottom left) and Na$_v$1.8-LI relative intensity (bottom right) are given. Neurons were considered positive if their Na$_v$1.8-LI was $\geqslant 20\%$ and negative if it was $< 20\%$. HTM, high threshold mechanoreceptive neurons; CV, conduction velocity; LY, Lucifer yellow; CB, Cascade blue.

Action potential overshoot. Recently it was shown (Renganathan *et al.* 2001) that the peak height of the AP was reduced in small DRG neurons in −/− neurons from $Na_v1.8$ knockout mice, compared with those from wild-type animals, indicating an important influence of $Na_v1.8$

on AP height. We therefore examined whether AP overshoot was related to $Na_v1.8$-LI relative intensity in neurons with membrane potentials equal to or more negative than −50 mV, with AP peaks that were at least −20 mV. There were indeed positive correlations between $Na_v1.8$-LI intensity and AP overshoot for all units together, for all nociceptive units, and for all positive units (Fig. 8).
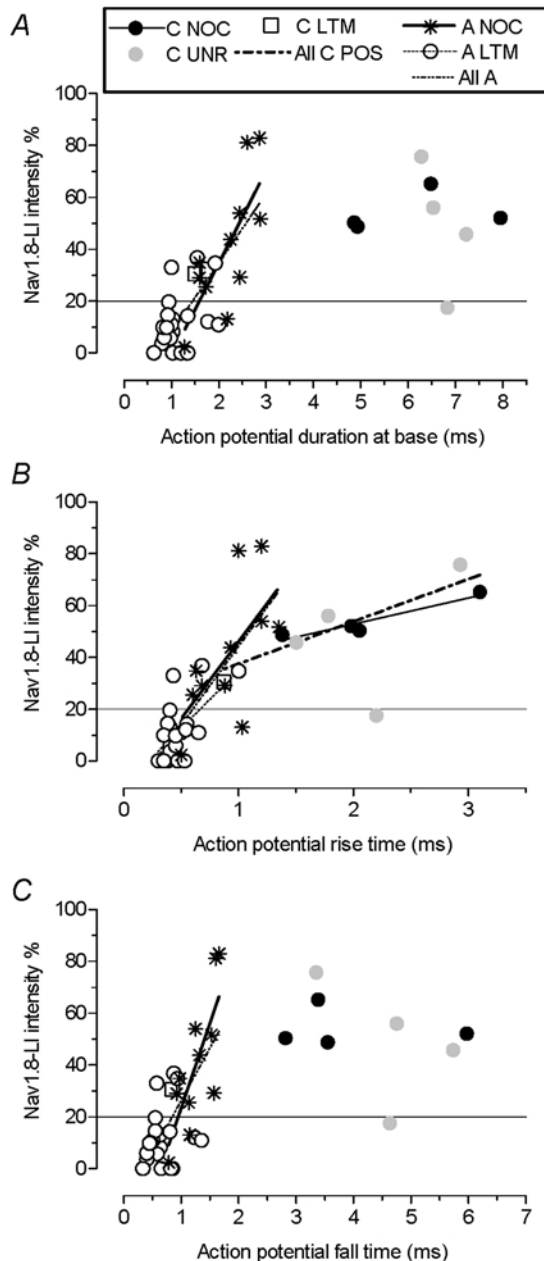
DISCUSSION

This is the first study to examine *in vivo* the properties of sensory neurons that express $Na_v1.8$. Moderately to strongly immunoreactive neurons were nociceptive with C-, Aδ- or Aα/β-fibres, and tended to have broad APs with large overshoots. Units with low, but clearly positive, immunoreactivity included both nociceptive and some LTM neurons with C-, Aδ or Aα/β-fibres.

There was clear cytoplasmic but not clear membrane $Na_v1.8$-LI in DRG neurons as previously described (Novakovic *et al.* 1998). This is not surprising as the density of Na^+ channels in the membrane required for the peak Na^+ current densities in DRG neurons are considerably below immunocytochemically detectable levels as previously discussed (Djouhri *et al.* 2003). As previously discussed (see Djouhri *et al.* 2003), there is evidence that the amount of channel in the cytoplasmic pool is much greater than that inserted into the membrane. The correlations between cytoplasmic immunoreactivity and membrane electrical properties (which presumably relate to the protein in the membrane)
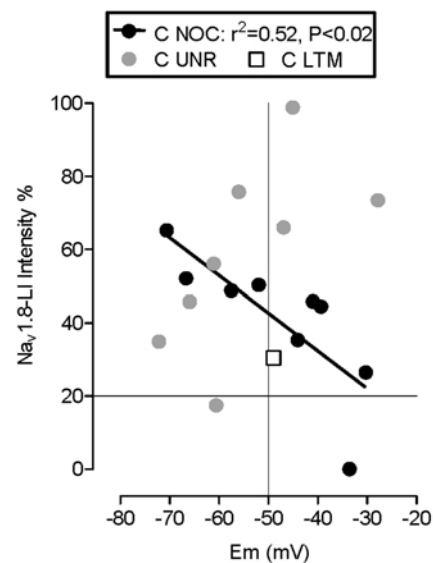

Figure 6. $Na_v1.8$-LI intensity and action potential shape

Only units with membrane potentials of at least −50 mV except the sole C-LTM (−48 mV) were included. Plots for AP duration at base (*A*), AP rise time (*B*) and AP fall time (*C*) are shown. Linear regressions were calculated for A-fibre nociceptors, A-fibre LTMs and all A-fibres together. Where regressions reached significance (*P* < 0.05) regression lines are shown, except for all units together (omitted for clarity). *P* and r^2 values for these and for other correlations are given in Table 1. In addition, there was a significant correlation for C-fibre nociceptor AP rise times (*P* < 0.05, r^2 = 0.91, *n* = 4). For abbreviations see legend to Fig. 4.

Figure 7. $Na_v1.8$-LI intensity and membrane potential

Membrane potentials of C-fibre neurons are plotted against $Na_v1.8$-LI intensity. A significant correlation was found for C-fibre nociceptive neurons but not for C-fibre unresponsive neurons. For abbreviations see legend to Fig. 4.

in this study appear to indicate some quantitative relationship between these two pools.

Neuronal size

Our finding of Na$_v$1.8 protein mainly in small–medium sized and but also in some large DRG neurons agrees with previous studies of mRNA and protein and with the presence of TTX-R$_s$ Na$^+$ current in mainly small–medium sized DRG neurons (see Introduction) and also in some large neurons (Sangameswaran *et al.* 1996; Tate *et al.* 1998; Novakovic *et al.* 1998; Coward *et al.* 2000; Renganathan *et al.* 2000).

Conduction velocity

The expression of Na$_v$1.8 protein in C-fibre neurons was expected. However, even the expression in A-fibre neurons is consistent with: (a) the medium to large size of some positive neurons; (b) TTX-R$_S$ current in large diameter afferents (Renganathan *et al.* 2000) which is likely to have A-fibres (Harper & Lawson, 1985); and (c) with Na$_v$1.8 expression in neurofilament-rich somata (Amaya *et al.* 2000) again indicating A-fibre CV (Lawson *et al.* 1984).

The correlations between CV and Na$_v$1.8-LI may imply a functional link between the two. Although not detected in normal rat peripheral nerve fibres (Novakovic *et al.* 1998), the amount required for AP conduction may not be immunocytochemically detectable (see above). The evidence that this channel is transported along fibres includes: (a) its detection in fibres in human normal peripheral nerve (Coward *et al.* 2000); (b) its presence in dorsal horn (Novakovic *et al.* 1998; Amaya *et al.* 2000); and (c) TTX-R Na$^+$ currents (presumably Na$_v$1.8 related) in peripheral terminals of afferent nerve fibres (Brock *et al.* 1998). In addition, TTX-R currents (presumably also Na$_v$1.8 related) can contribute to AP conduction, because some C-fibre conduction is TTX resistant (Jeftinija, 1994; Quasthoff *et al.* 1995; Kobayashi *et al.* 1996; Buchanan *et al.* 1996; Steffens *et al.* 2001). The absence of a specific blocker for TTX-R$_S$ currents means that an important contribution to conduction by these currents, where present, cannot be ruled out even where conduction is blocked by TTX.

Although CV was not correlated with Na$_v$1.8-LI intensity in C-fibre somata, this does not exclude a role of Na$_v$1.8 in conduction along C-fibres (see above). It does, however, appear from the correlation between Na$_v$1.9 immunoreactivity and C-fibre CV, that Na$_v$1.9 may have an influence on CV in these unmyelinated fibres (Fang *et al.* 2002). In contrast to the C-fibres, the clear correlation between Na$_v$1.8 and CV in A-fibres may result from the presence of TTX-S$_F$ currents in A-fibre neurons with much faster kinetics than the TTX-R$_S$ current associated with Na$_v$1.8. If more available Na$_v$1.8 is accompanied by less TTX-S$_F$ current, this could account for units with more

available Na$_v$1.8 having slower AP rise times, leading to slower CVs.

C- and A-fibre nociceptive neurons

We found Na$_v$1.8 in nociceptive neurons with C-fibres as expected (Introduction). The presence of Na$_v$1.8 in nociceptive A-fibre neurons was also consistent with previous descriptions of TTX-R$_S$ Na$^+$ currents in these neurons (Ritter & Mendell, 1992).

The Na$_v$1.8-LI in nociceptors and its even higher intensity in C-unresponsive neurons are interesting in view of the suggestion that the high activation threshold of Na$_v$1.8 (Sangameswaran *et al.* 1996; Waxman, 1999) may contribute to the high thresholds of nociceptive neurons (Khasar *et al.* 1998; Akopian *et al.* 1999). Since C-unresponsive neurons probably include very high threshold 'silent' nociceptive neurons (see Methods), their high levels of Na$_v$1.8 may contribute to this high threshold.

Nociceptors *versus* low threshold mechanoreceptive units

The labelling of both nociceptive and some LTM neurons in our study accords with Na$_v$1.8-LI distribution in superficial (I and II) and deeper (III–V) laminae of the dorsal horn (Amaya *et al.* 2000). The TTX-R$_S$ Na$^+$ current described in half the large cutaneous afferent neurons (Renganathan *et al.* 2000) could be explained by Na$_v$1.8 being in 60 % of Aα/β-fibre nociceptive and in 15–20 % of LTM Aα/β-fibre cutaneous afferent neurons (this study). Nonetheless, the intensity of Na$_v$1.8 was much greater in
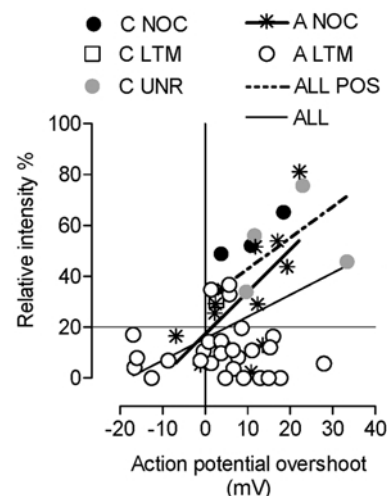

Figure 8. Na$_v$1.8-LI relative intensity *versus* action potential overshoot

Only units with membrane potentials of at least −50 mV except the sole C-LTM (−48 mV) and somatic APs with peaks that reached at least −20 mV at peak were included. Regression lines are shown where there was a significant ($P < 0.05$) linear regression. *P* and r^2 values for these correlations were: for all A-fibre units, $P < 0.0001$, $r^2 = 0.56$; for all positive units, $P < 0.0001$, $r^2 = 0.56$; for A-fibre nociceptive units $P < 0.0001$, $r^2 = 0.42$. For abbreviations see legend to Fig. 4.

nociceptive than LTM neurons; indeed unlike nociceptors, LTMs were never more than weakly positive. The proportion of A LTM neurons that was positive was low, and $Na_v1.8$-LI was absent in muscle spindle afferent neurons consistent with their absence of TTX-R currents (Honmou *et al.* 1994). Thus despite its detectable presence in some LTMs, its low levels in these neurons may indicate a smaller influence of this channel on LTM than on nociceptive membrane properties.

$Na_v1.8$-LI and action potential duration

Estimates of the contribution of $Na_v1.8$-related TTX-R_S current to APs in small DRG neurons include ~45 % of total peak Na^+ current density (Akopian *et al.* 1999), 60 % of total Na^+ channel conductance (Novakovic *et al.* 1998) and about 60 % (Blair & Bean, 2002) or up to 80–90 % of the inward Na^+ current during the rising phase of the AP (Renganathan *et al.* 2001). Compared with TTX-S Na^+ currents, TTX-R_s currents in sensory neurons have slower rates of activation and much slower rates of inactivation (e.g. Kostyuk *et al.* 1981; Elliott & Elliott, 1993; Schild & Kunze, 1997). Therefore greater availability of $Na_v1.8$ should slow the AP kinetics. This effect may be more evident across a group of neurons many of which have APs generated largely by the faster TTX-S current, namely the larger diameter faster conducting neurons, which could account for the stronger correlation of $Na_v1.8$ with AP duration in A- than C-fibre neurons.

In positive C-fibre neurons the correlation between $Na_v1.8$-LI intensity and AP rise time but not fall time or overall duration is similar to the finding for $Na_v1.9$-LI (Fang *et al.* 2002). Interestingly in A-fibre neurons the correlation with rise time was also stronger than with AP fall time. This may indicate a greater influence of $Na_v1.8$ on rise than fall time, perhaps because the rising phase of the AP must depend almost entirely on the availability and kinetics of Na^+ channels, whereas the fall time is probably also influenced by other types of current such as Ca^{2+} current (e.g. see Blair & Bean, 2002) and K^+ currents.

Broad/inflected APs are characteristic of nociceptive DRG neurons *in vivo* (see Introduction). Thus, if greater $Na_v1.8$ in the soma results in greater TTX-R_S AP inward current, the presence of $Na_v1.8$ in nociceptors could contribute to their broader APs. However, other Na^+ channels such as $Na_v1.9$ (see above) and $Na_v1.7$-LI (Djouhri *et al.* 2003), also found preferentially in smaller DRG neurons, have immunoreactivity correlated with AP duration. Thus all three may contribute to the characteristic broad AP of nociceptive neurons either directly (e.g. $Na_v1.8$ because of its slow kinetics) or indirectly (perhaps via the postulated depolarising effect of $Na_v1.9$ persistent current (Dib-Hajj *et al.* 2002) inactivating low threshold TTX-S Na^+ channels (e.g. $Na_v1.7$), and thus slowing AP rise time (Djouhri *et al.* 2003). The strong correlation of $Na_v1.9$ with AP rise time

and CV (Fang *et al.* 2002) may indicate that this channel has a dominant influence on these in C-fibre neurons, while the stronger correlation of $Na_v1.8$ with AP duration and CV in A-fibre neurons may result from the probably smaller influence of $Na_v1.9$, allowing the slow kinetics of $Na_v1.8$ to have a greater influence.

The correlation between AP overshoot and $Na_v1.8$-LI was consistent with the findings of Renganathan *et al.* (2001) indicating an influence of $Na_v1.8$ on AP height. This may result from the slow kinetics of TTX-Rs current associated with $Na_v1.8$, which may enable the membrane potential to approach more closely the Na^+ equilibrium potential than channels with faster kinetics. It may therefore be the presence of $Na_v1.8$ that leads to the consistently greater overshoot in nociceptive than LTM DRG neurons of all CV ranges (Djouhri & Lawson, 2001*a*).

Conclusions

$Na_v1.8$ was present in most C- and A-fibre nociceptive neurons and C-fibre unresponsive (nociceptor type) neurons. In contrast detectable immunoreactivity was not found in muscle spindle, nor in most cutaneous $A\alpha/\beta$ LTM units and only weak positive labelling was found in other LTM units. The finding that $Na_v1.8$-LI intensity was correlated with properties associated with nociceptive function, namely long AP durations (especially AP rise time) and large AP overshoots (Lawson, 2002), supports the previous suggestion (Akopian *et al.* 1996) that $Na_v1.8$ may be important in influencing the membrane properties of nociceptors

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