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Sensory and electrophysiological properties of guinea-pig sensory neurones expressing Na$_v$1.7 (PN1) Na$^+$ channel α subunit protein

Laiche Djouhri*, Richard Newton*, Simon Rock Levinson†, Carol M. Berry*, Barbara Carruthers* and Sally N. Lawson*

* *Department of Physiology, Medical School, Bristol BS8 1TD, UK and* † *University of Colorado Health Sciences Center, Denver, C0 80262, USA*

The TTX-sensitive Na$_v$1.7 (PN1) Na$^+$ channel α subunit protein is expressed mainly in small dorsal root ganglion (DRG) neurones. This study examines immunocytochemically whether it is expressed exclusively or preferentially in nociceptive primary afferent DRG neurones, and determines the electrophysiological properties of neurones that express it. Intracellular somatic action potentials (APs) evoked by dorsal root stimulation were recorded in L6/S1 DRG neurones at 30 ± 2 °C *in vivo* in deeply anaesthetised young guinea-pigs. Each neurone was classified, from its dorsal root conduction velocity (CV) as a C-, Aδ- or Aα/β-fibre unit and from its response to mechanical and thermal stimuli, as a nociceptive, low threshold mechanoreceptive (LTM) or unresponsive unit. Fluorescent dye was injected into the soma and Na$_v$1.7-like immunoreactivity (Na$_v$1.7-LI) was examined on sections of dye-injected neurones. All C-, 90 % of Aδ- and 40 % of Aα/β-fibre units, including both nociceptive and LTM units, showed Na$_v$1.7-LI. Positive units included 1/1 C-LTM, 6/6 C-nociceptive, 4/4 C-unresponsive (possible silent nociceptive) units, 5/6 Aδ-LTM (D hair), 13/14 Aδ-nociceptive, 2/9 Aα/β-nociceptive, 10/18 Aα/β-LTM cutaneous and 0/9 Aα/β-muscle spindle afferent units. Overall, a higher proportion of nociceptive than of LTM neurones was positive, and the median relative staining intensity was greater in nociceptive than LTM units. Na$_v$1.7-LI intensity was clearly positively correlated with AP duration and (less strongly) negatively correlated with CV and soma size. Since nociceptive units tend overall to have longer duration APs, slower CVs and smaller somata, these correlations may be related to the generally greater expression of Na$_v$1.7 in nociceptive units.

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Corresponding author S. N. Lawson: Department of Physiology, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK. Email: sally.lawson@bristol.ac.uk

Voltage-gated Na$^+$ channels are important for generation and conduction of action potentials (APs). They are composed of α subunits that form the voltage-sensitive and ion-selective pore, and β subunits that can modulate the properties of the α subunit (see Catterall, 2000). Na$^+$ channel α subunits in dorsal root ganglia (DRGs) include tetrodotoxin-resistant (TTXR) and TTX-sensitive (TTXS) channel subunits. The TTXS α subunit Na$_v$1.7 (PN1 or peripheral nerve type 1), present in DRG tissue (Sangameswaran *et al.* 1997; Toledo-Aral *et al.* 1997) is the rat homologue of both the human neuroendocrine Na$^+$ channel (hNE) (Klugbauer *et al.* 1995) found in adrenal and thyroid glands, and the rabbit Na$^+$ channel NaS (Belcher *et al.* 1995).

Na$_v$1.7 protein is expressed in DRG and sympathetic ganglion neurones (Toledo-Aral *et al.* 1997). Na$_v$1.7 mRNA is at higher levels in the peripheral than the central nervous system, with some studies finding no Na$_v$1.7/Na$_v$1.7 mRNA in the rat CNS (Klugbauer *et al.* 1995; Toledo-Aral *et al.* 1997). Thus, while not exclusively

located in DRG neurones, Na$_v$1.7 is much more highly expressed in these than in CNS neurones. Despite the distribution of Na$_v$1.7 mRNA in DRG neurones of all sizes (Black *et al.* 1996), anti-Na$_v$1.7 antibodies show more intense labelling of small than large DRG neurones in adult (Porreca *et al.* 1999, Gould *et al.* 2000) but not in fetal rats (Toledo-Aral *et al.* 1997). Thus Na$_v$1.7 protein, but not mRNA appears to be more highly expressed in small than large adult DRG neurones.

It is important to determine which Na$^+$ channel subunits are restricted to, or preferentially expressed in, nociceptive neurones, since such subunits may prove to be useful targets for novel analgesics. Small DRG neurones are often assumed to be nociceptive. Interest has therefore been focussed on Na$^+$ channel α subunits that are expressed preferentially in these neurones. These include the TTXR subunits Na$_v$1.8 (SNS/PN3) (Akopian *et al.* 1996; Tzoumaka *et al.* 1997) and Na$_v$1.9 (NaN/SNS2) (Dib-Hajj *et al.* 1998; Tate *et al.* 1998) and the TTXS subunit protein Na$_v$1.7. However, since cell size alone is an unsafe

predictor of nociceptive function (S. N. Lawson, unpublished observations, also see Hoheisel *et al.* 1994), direct examination of sensory properties is essential to establish whether $Na_v1.7$ protein in DRGs is limited to, or preferentially expressed in, nociceptive neurones.

APs in small sized DRG neurones have Na^+ inward currents with both TTXR and TTXS components. The TTXR inward current in the AP is thought to be via the $Na_v1.8$ channel subunit (Akopian *et al.* 1996), and although $Na_v1.7$ is thought to be involved in impulse initiation (Cummins *et al.* 1998), its contribution to fibre conduction velocity (CV) and to the inward current in somatic APs is not clear.

We have therefore examined in DRG neurones (a) whether detectable $Na_v1.7$-LI is only in, or is more intense in, nociceptive neurones and (b) whether $Na_v1.7$-LI levels in neuronal somata are related to active membrane properties of somata or fibres. To achieve this, we have made intracellular voltage recordings from individual DRG neurones in anaesthetised guinea-pigs *in vivo*, identified their sensory properties and passed fluorescent dye into their somata. We then determined immunocytochemically the $Na_v1.7$-LI of these identified neurones.

An abstract referring to preliminary data included in this paper has been published (Djouhri *et al.* 2000).

METHODS

All experimental procedures used conformed with the UK Animals (Scientific Procedures) Act 1986.

Guinea-pigs were prepared for electrophysiological recordings as previously described (Djouhri *et al.* 1998) and sensory properties of units were established as described in full in Lawson *et al.* (1997). Briefly the methods were as follows. Young female guinea-pigs (weight 160–300 g) were deeply anaesthetised with sodium pentobarbitone with an initial dose of 50 mg kg^{-1}, I.P. A tracheotomy allowed artificial ventilation and end-tidal CO_2 monitoring. Further anaesthetic in doses of 10 mg kg^{-1} as needed (about every hour) to maintain areflexia (tested regularly with pinch of the forepaw), was given via a cannula in the left carotid artery. In later experiments, blood pressure was monitored throughout the experiment.

The ilium and the L1 vertebra were clamped. A laminectomy exposed L6 and S1 DRGs. A small silver platform beneath the DRG increased stability during recording. Adipose tissue over the DRG was removed, taking care not to damage the DRG surface blood vessels. The dorsal root of the DRG under study was cut as close as possible to the spinal cord and a pair of bipolar platinum electrodes inserted under it. This was used for (a) establishing, with extracellular recording, the receptive field region of the whole DRG to natural stimulation and (b) stimulation of the dorsal root during intracellular recordings. Recordings were made in a paraffin pool maintained at 28.0–31.5 °C.

Intracellular recordings

Just before recording, a muscle relaxant was administered intra-arterially (I.A.) accompanied always by an additional dose (10 mg kg^{-1}, I.A.) of the anaesthetic. The muscle relaxant used was either gallamine triethiodide (Flaxedil, 2 mg kg^{-1} I.A.) or pancuronium (0.5 mg ml^{-1} I.A.). These same doses of muscle relaxant and anaesthetic (always given together) were administered regularly (approximately hourly). The frequency and amount of anaesthetic administered during the neuromuscular blockade was the same as that required to maintain complete areflexia in the 2 h prior to administering the Flaxedil. Blood pressure recordings in later experiments of the series with this anaesthetic regime were very stable throughout the experiment, indicating deep anaesthesia.

Intracellular recordings from DRG somata were made with glass microelectrodes filled with a fluorescent dye. The fluorescent dyes were Lucifer yellow CH (LY; approx. 5 mg ml^{-1} in 0.1 M LiCl solution; 100–700 MΩ, mean ± S.D. = 360 ± 154 MΩ), ethidium bromide (EB; 6 mM in 1 M KCl; 60–509 MΩ, mean 127 ± 89 MΩ), or occasionally Cascade Blue (CB) at 30 mg ml^{-1} in 0.1 M LiCl solution (260–600 MΩ, mean 455 ± 120 MΩ). The microelectrode was advanced until a membrane potential was seen and an AP could be evoked by dorsal root stimulation with single rectangular pulses with durations of 0.03 ms (for A-fibre units) or 0.3 ms (for C-fibre units). Stimulus intensity was adjusted to twice threshold for A-fibre units and suprathreshold (usually 1–1.5 times threshold) for C-fibre units, and somatic APs were recorded on-line with a CED (Cambridge Electronics Design, Cambridge, UK) 1401 plus interface and the SIGAV program from CED and were subsequently analysed with a script running in the Spike II program (CED). The AP parameters measured were described previously (Djouhri *et al.* 1998) and include the AP duration at base (AP duration). The membrane potential was also recorded.

Conduction velocity (CV) was estimated from the latency to the rise of the dorsal root-evoked somatic AP, and the conduction distance (typically 4–7 mm) from the approximate (± 0.25 mm) location of the neurone in the DRG. The maximum measurement error of conduction distance was 10–15 % and utilisation time was not taken into account (for more details see Djouhri *et al.* 2001). CV ranges (C, Aδ and Aα/β) were based on dorsal root compound action potential waves (Djouhri *et al.* 1998), as follows: C < 1.1 m s^{-1}, Aδ 1.1–4.2 m s^{-1} and Aα/β > 4.2 m s^{-1}. Reasons for the relatively low CVs have been discussed previously (Lawson *et al.* 1997; Djouhri *et al.* 1998).

Sensory receptive properties

The sensory receptive fields of DRG neurones were on the ipsilateral hind limb and flank; their properties were examined and classified as previously described (Lawson *et al.* 1997; Djouhri *et al.* 1998). To summarise, units that responded to low intensity mechanical stimulation were categorised as low threshold mechanoreceptive (LTM) units. Most of these were cutaneous but Aα/β-fibre units with deeper receptive fields that responded to light pressure against muscle tissue, followed vibrations of 100 or 250 Hz, and often showed ongoing discharges, were classified as muscle spindle (MS) afferent units. This latter group included both Group I (Aα) and Group II (Aβ) categories and may have also included some Golgi tendon organ afferents. Low threshold C-fibre mechanoreceptive (C-LTM) units responded preferentially to slow gentle contact moving across the skin

at < 1 mm s^{-1} and sometimes to cooling. Units that did not respond to low intensity stimuli but did respond to strong mechanical stimuli and lacked prompt responses to noxious heat were classified as high threshold mechanoreceptive (HTM) units. Some nociceptive units responded both to high intensity mechanical stimuli and also promptly to a single application of hot water at $> 50\,^{\circ}$C (noxious heat); these included A-mechano-heat (A-MH) units; C-polymodal (C-PM) units with superficial (probably epidermal or superficial dermal) receptive fields and C-mechanoheat (C-MH) units with receptive fields that were not superficial (presumed to be in dermis). The term 'nociceptive units' in this paper includes HTM as well as MH or C-PM units. Cooling units were those that responded to a brief spray of ethyl chloride, a cooled metal rod or ice and that often showed ongoing activity at room temperature, which ceased with slight warming.

Unresponsive units with A- or C-fibres were those for which no receptive field was found despite an extensive search over the whole hind limb with the usual non-noxious and noxious, mechanical and thermal stimuli.

Neuronal labelling
Once each unit was characterised, dye was ejected electro-phoretically into the soma from the electrode by rectangular current pulses (1 nA for 500 ms at 1 Hz) for periods of up to 5 min. These currents were negative for LY and CB (0.6–1.0 nA min) and positive for EB (0.3–1 nA min). Usually two neurones at opposite ends of each DRG were injected with LY or CB, and one neurone in the mid-region was marked with EB. In this study, 44 cells were labelled with LY, 29 with EB and 5 with CB.

At the end of the experiment, the animals were killed under deep anaesthesia by perfusion through the heart with 0.9 % saline followed by Zamboni's fixative (Stefanini *et al.* 1967). Experimental DRGs were removed, post-fixed in Zamboni's, and soaked in 30 % sucrose buffer at 4 $^{\circ}$C overnight. The next day, serial 7 μm cryostat sections of the DRGs were cut. Sections were mounted on 20 slides so that each slide had a series made up of every 20th section. Each section was examined under fluorescence microscopy; digital images of profiles of dye-labelled neurones were captured and camera lucida drawings made to ensure relocation of each profile after immunocytochemistry. The detailed position of every dye-filled cell recovered was compared to cell locations recorded during the experiment. If the expected and actual locations did not match, or if too many or too few cells were labelled, units were rejected. Tissue sections were stored at $-20\,^{\circ}$C until immunocytochemistry was carried out. Small 'edge' sections through a dye-labelled cell, that might not include the cell throughout the section thickness, were not used for immunocytochemistry.

Rat tissue
Cryostat sections (7 μm) of DRGs from rats that had been perfuse-fixed with Zamboni's fixative or 4 % paraformaldehyde under deep pentobarbitone anaesthesia were used for immunocytochemistry on rat tissue.

Immunocytochemistry
Endogenous biotin-like activity was blocked using the Vector SP-2001 blocking kit, prior to immunocytochemistry. Avidin–biotin immunocytochemistry (Vectastain Elite ABC kit: rabbit IgG, Vector Laboratories Ltd, UK) was performed, adding 0.3 % Triton X-100 to the washes, antibody and ABC steps. Sections were exposed to primary anti-Na$_v$1.7 antibody at a concentration of 3 μg ml^{-1} in Tris-buffered saline with 0.3 % Triton X-100, for approximately 48 h at 4 $^{\circ}$C. Any sections with generally poor immunocytochemistry were not used for further analysis.

Characterisation of the anti-Na$_v$1.7 antibody on guinea-pig tissue
The primary polyclonal anti-Na$_v$1.7 antibody had been prepared as previously reported (Toledo-Aral *et al.* 1997) by immunising rabbits with a synthetic peptide coupled to keyhole limpet haemocyanin, to residues 446–460 in rat Na$_v$1.7, a sequence not present in other Na$^+$ channel subunits. Because the antibody was raised against an epitope on rat Na$_v$1.7, and the original antibody characterisations were done on rat tissue, we examined whether the staining in the guinea-pig was similar and therefore likely also to be specific for Na$_v$1.7. In addition, the specificity of the antibody for Na$_v$1.7 in guinea-pig DRG tissue was examined with preabsorption tests, using the peptide against which the antibody was raised, as follows. Sections from the same guinea-pig DRG were treated identically except for the primary antibody incubation, which was with one of the following: (a) antibody (3 μg ml^{-1}) that had not been preabsorbed was applied to the section overnight at 4 $^{\circ}$C (Fig. 1*C*); (b) Na$_v$1.7 antibody (3 μg ml^{-1}) preabsorbed by incubating overnight with peptide at a concentration of 5×10^{-5} M, representing a 100 times molar excess of peptide over antibody, and then applied to the section overnight at 4 $^{\circ}$C (Fig. 1*D*) or (c) no primary antibody (Fig. 1*E*). In addition, Western blots were carried out on both guinea-pig and rat DRG tissue with this antibody. Further confirmation of the specificity of this antibody was achieved by comparing staining patterns with a separate polyclonal antibody raised to a different epitope on Na$_v$1.7.

Analysis of Na$_v$1.7-LI relative intensity
Dye-injected neurones stained to show Na$_v$1.7-LI were each scored in two ways. The first scoring method was subjective by agreement between two observers as follows: 0 means negative, and 1–5 means clearly positive; 1 means weakly positive and 5 is equal to the most intensely stained neurone seen in the section. Secondly, a more objective semi-quantitative method of assessing relative intensity using image analysis was used. Images of cells were captured under standardised brightfield conditions using Openlab software (Improvision, Coventry, UK) and a cooled integrating CCD camera (Photonic Science, Robertsbridge, UK), and analysed with a program (macro) to run in NIH image. For each cell cytoplasm (excluding the nucleus), the mean value of the darkest 10 % of the pixels (from a frequency distribution histogram of pixel density) was taken as a measure of absorbance of light by the Na$_v$1.7-LI reaction product. This method enables labelling that is punctate or globular to be clearly distinguished above background levels. Using these measures, the relative intensity (absorbance level) for each dye-injected cell was calculated as follows. To provide comparisons with the dye-labelled cell, the 0 % intensity level (*a*) was taken as the mean intensity of three clearly negative neuronal profiles near the labelled cell. The 100 % intensity level (*b*) was the mean intensity of the three most intensely stained profiles in the section. The intensity of the dye-labelled cell provided the value '*c*'. The relative Na$_v$1.7-LI intensity of the cell was calculated as $(c - a)/(b - a)$ and expressed as a percentage. The calculated relative intensities and subjective relative intensity 0–5 ratings were highly correlated with an r^2 value of 0.91, $n = 78$ (Fig. 1*F*). Because neurones with relative intensity > 20 % were consistently judged subjectively as
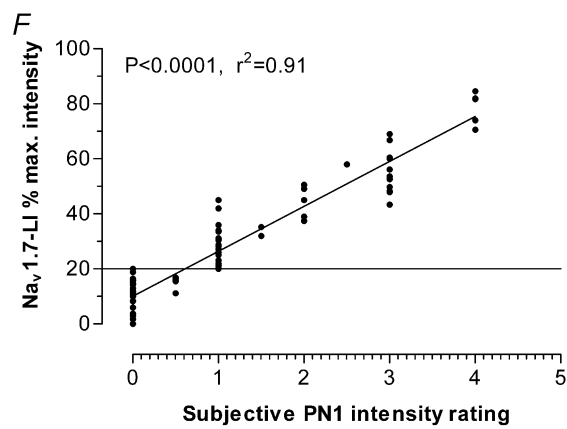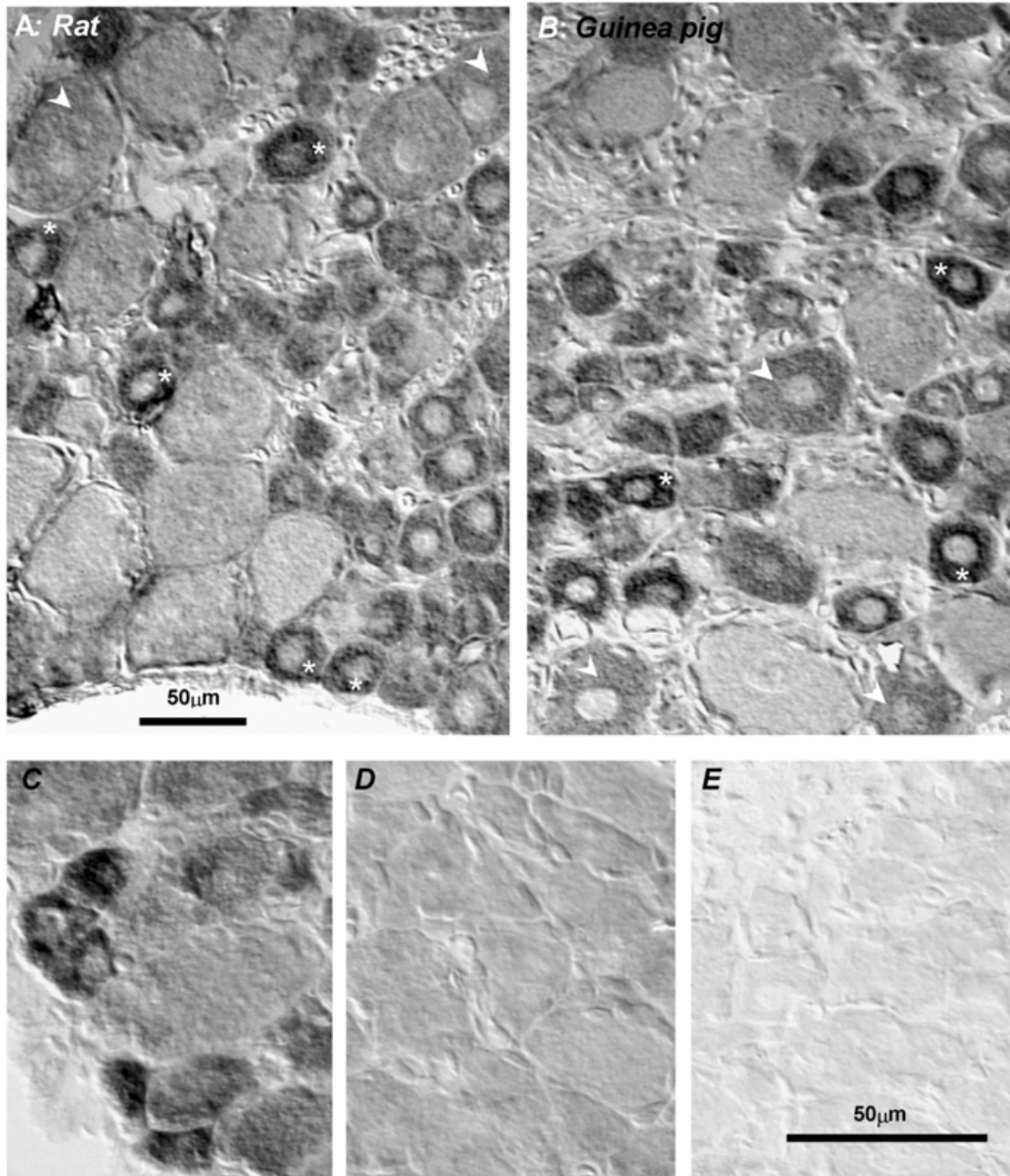
Figure 1. For legend see facing page.

being clearly positive by all viewers (see Fig. 1*F*), the term 'positive' refers to neurones with relative intensities > 20 %, and 'negative' refers to units with values < 20 %. We cannot exclude the possibility that cells with relative intensities < 20 % and classified as 'negative' may have had low levels of Na_v1.7 protein. This method compensates for variability in immunocytochemical staining between sections and/or different immunocytochemical runs.

Cell size measurement

For each dye-labelled neurone, the cross sectional area of all the sections was measured and the largest of these sections (usually containing a clear nuclear profile) was used as the measure of cell size. Although this would be a perfect indicator of size for completely spherical neurones, not all neurones are spherical.

Sampling bias

The ease of making recordings from these neurones is greater for faster conducting neurones. This was offset to some extent by a conscious bias towards selecting neurones with slower CVs and/or nociceptive receptive fields. Nonetheless, the proportion of C-fibre neurones is considerably under-represented in this study.

Criteria for acceptance of data

Samples of each (sensory) type of DRG neurone were chosen for immunocytochemistry. This and the sampling bias mean that the proportions of cells in each group do not therefore provide an accurate indication of the proportions of different types of unit in the DRG. For the 78 units with successful immunocytochemistry, the mean animal weight was 220 ± 3.6 g (s.e.m.), range 167–300 g and the mean temperature at the DRG was 30 ± 0.12 °C (s.e.m.).

For the analysis of AP characteristics, cells were included only if (a) they had membrane potentials of −40 mV or more negative; (b) the temperature at the DRG was 28–31.5 °C; and (c) the somatic AP had an amplitude > 35 mV. The number of cells included in this analysis was 68.

Statistics

2 × 2 contingency tables and χ^2 tests compared proportions of neurones (Fig. 3). The median relative intensities of groups shown in Fig. 4, except for the single C-LTM unit, were compared with the Kruskall–Wallis test (the non-parametric equivalent of one way ANOVA) followed by Dunn's *post hoc* test comparing all pairs of groups (Prism version 3, GraphPad Software Inc., USA). The median relative intensities of nociceptive and LTM units (Fig. 4) were compared with the Mann-Whitney test using Prism Software; linear regression analysis carried out on the data displayed in Fig. 5 is shown in Table 1. Where significant correlations existed on the same graph e.g. for nociceptors and

LTM units, the regression lines were compared (Table 1) for slope, intercepts and elevation (Prism 3 software, also see Zar, 1984).

Possible sources of error

The problems with locating dye-injected cells have been reported in detail (Lawson *et al.* 1997), and all precautions necessary to avoid the problem of cells being inadvertently labelled by dye leakage from the electrode that were outlined in that paper were followed in the present study. The weight range of the animals and the temperature at the DRG were controlled within narrow limits. Minor sources of error in CV are described above.
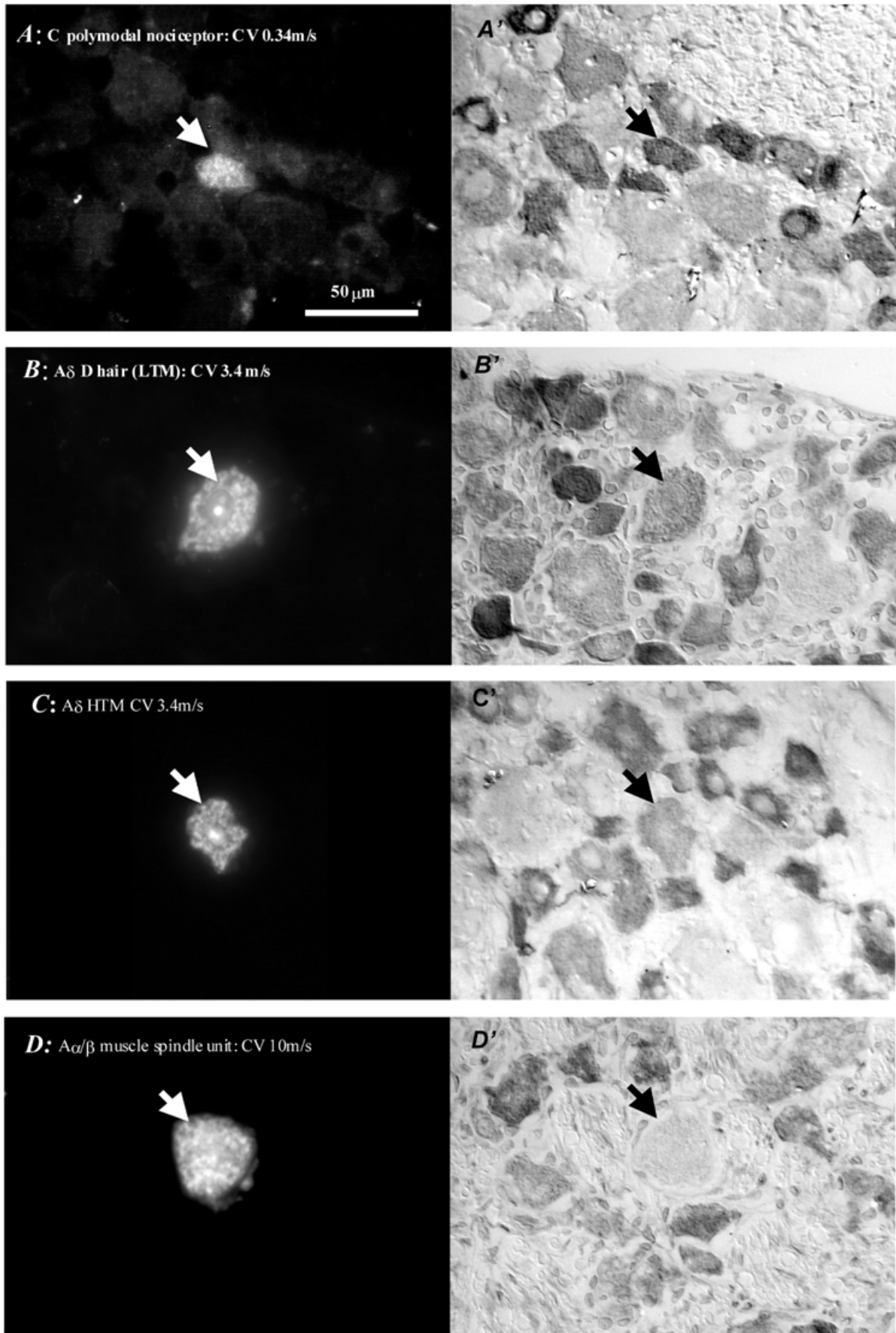
RESULTS

The staining pattern in the rat (Fig. 1*A*) was indistinguishable from that in the guinea-pig (Fig. 1*B*), with smaller DRG neurones being more intensely stained than large neurones, and with some large neurones weakly stained or unstained. Preabsorption of the anti-Na_v1.7 antibody led to virtually complete loss of staining, leaving a level of background staining that was somewhat higher than when no primary antibody was included, both in guinea-pig (Fig. 1*C*, *D* and *E*) and rat DRG sections (not shown). This Na_v1.7 antibody specifically detected a protein of an appropriate size following Western blot analysis of rat DRG membrane extract (Toledo-Aral *et al.* 1997). A similar result was obtained here with Western blots (not shown) of both guinea-pig and rat DRG protein preparations. In addition, another Na_v1.7 antibody raised against a different epitope showed similar staining to the present antibody in rat DRGs, confirming the preferential small cell labelling. There is therefore good evidence that the antibody we used labels Na_v1.7 protein, preferentially in smaller neurones, in both rat and guinea-pig DRGs.

Na_v1.7-LI was examined in 78 DRG neuronal somata with identified sensory properties. These included 12 C-fibre units (6 nociceptive, 1 C-LTM, 4 unresponsive and 1 C-cooling units), 27 Aδ-fibre units (14 nociceptive, 6 D hair LTM, 1 cooling unit (see below), 1 moderate pressure that also fired to cooling and 5 unresponsive units), and 39 Aα/β-fibre units (9 nociceptive, 27 LTM units including 9 muscle spindle and 18 cutaneous LTMs, and 3 unresponsive units). The Aδ moderate pressure unit was not included in nociceptive or LTM groups as it had

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Figure 1. Immunostaining of neurones to show Na_v1.7 in rat and guinea-pig DRGs

A and *B* compare the staining with anti-Na_v1.7 antibody of rat (*A*) and guinea-pig (*B*) DRG sections on the same slide. The small cells were the most intensely stained (asterisks), medium sized cells often had an intermediate staining intensity (arrowheads) and the palest staining profiles were usually of large neurones. These images were captured under bright field optics with interference contrast to aid visualisation of the paler staining neurons, although image analysis was always carried out on bright field images. *C*, *D* and *E*, sections of the same guinea-pig L6 DRG on which immunocytochemistry was carried out simultaneously with the following primary antibody: *C*, normal anti-Na_v1.7 antibody; *D*, anti-Na_v1.7 antibody was pre-absorbed for 3 h prior to use with 5×10^{-5} M of the peptide against which the antibody had been generated; *E*, no primary antibody. *F*, the relationship between subjectively judged relative intensity (abscissa) and the calculated relative intensity (ordinate) (see Methods); 20 % relative intensity or above was consistently judged to be clearly positive.

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A: C polymodal nociceptor: C V 0.34 m/s

A'

50 μm

B: Aδ D hair (LTM): C V 3.4 m/s

B'

C: Aδ HTM CV 3.4 m/s

C'

D: Aα/β muscle spindle unit: CV 10 m/s

D'
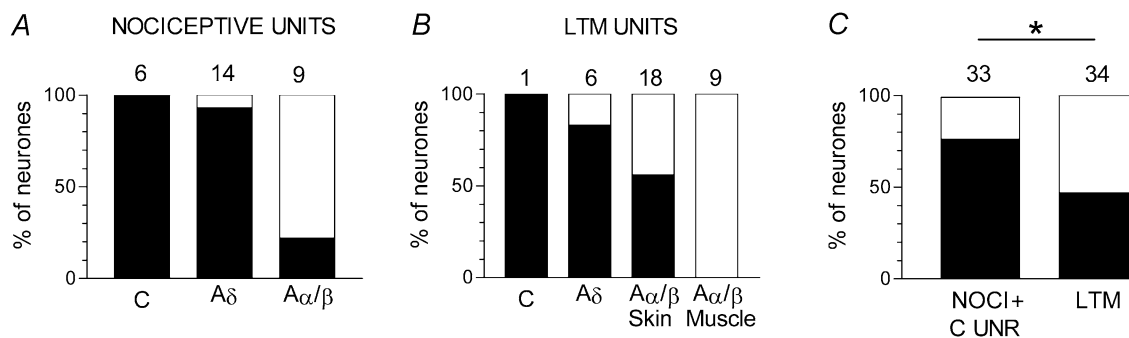
For legend see facing page.

Figure 3. Comparison of the percentages of neurones with different CVs and sensory properties that show Na$_v$1.7-LI

Graphs show the percentages of nociceptive (*A*) and LTM (*B*) units in the different CV ranges, and in nociceptive and LTM (low threshold mechanoreceptive) units in all CV groups together (*C*) that showed clear Na$_v$1.7-LI (≥ 20 % relative intensity). It can be seen in *A* and *B* that these percentages were lower in groups with higher mean CVs. *C*, the percentage of nociceptive units that was positive was significantly greater than the percentage of LTM units that was positive (χ^2 test, 2 × 2 contingency table). The number of units is shown above each column. NOCI, nociceptive, all conduction velocities; C UNR, C-fibre unresponsive units.

sensory receptive and electrophysiological properties intermediate between the two. The cooling Aδ unit fired spontaneously at room temperature, and responded to a cooled metal spatula applied to hairy skin but not to purely mechanical stimuli. The C-fibre unresponsive units had very long AP and AHP durations, indicating (see Djouhri & Lawson, 1998) that they were 'nociceptive-type' neurones, likely to be either very high threshold nociceptive neurones (the so-called 'silent nociceptors') or nociceptive units with inaccessible receptive fields. However, many of the A-fibre unresponsive neurones had AP and AHP durations consistent with some of them being LTM units with inaccessible receptive fields. These A-fibre unresponsive units were therefore not included in later analyses, as their identity was unclear. Examples of Na$_v$1.7-LI in identified units in Fig. 2 include a C-PM unit with intense staining, two Aδ units both with moderate staining intensities (a D hair LTM and an Aδ-nociceptive unit) and a muscle spindle afferent unit which is negative (< 20 % relative absorbance).

Of the 68 units that fulfilled the criteria for the AP analyses, 10 were C-fibre units (5 nociceptive, 1 C-LTM, 1 C cooling and 3 unresponsive), 24 were Aδ-units (14 nociceptive, 4 D hair LTM, the moderate pressure unit that fired to

cooling and 5 unresponsive units), and 34 were Aα/β units (7 nociceptive, 24 LTM units and 3 unresponsive units).

Comparisons of the proportions of units that were clearly positive (relative intensity ≥ 20 %) for Na$_v$1.7 are shown in Fig. 3. All C-, 93 % Aδ- and 22 % Aα/β-fibre nociceptive units were Na$_v$1.7-LI-positive. However, LTM units were also positive including one weakly positive C-LTM unit, most (83 %) D hair units, 56 % of the cutaneous Aα/β LTM units but none of the muscle spindle LTM units. Thus positive units included both nociceptive and LTM units. Overall, however, the proportion of all nociceptive units, including C-unresponsive (i.e. nociceptive-type) units, that showed Na$_v$1.7-LI (72.4 %) was significantly greater than the proportion of LTM units that was positive (47 %), Fig. 3*C*.

There is a wide range of relative intensities of Na$_v$1.7-LI in the different groups of DRG neurones (Fig. 4). Despite this range, statistical differences (Kruskall–Wallis test) existed between the medians of some groups, which were generally higher in the groups with slower CVs. The median for all nociceptive units was significantly greater than that for all LTM units (Mann–Whitney test). There was no clear difference in intensity between C-HTMs and

Figure 2. Photomicrographs of dye-injected, physiologically identified neurones

On the left are the fluorescence photomicrographs of sections through neurones indicated by the arrow in each image injected with Lucifer yellow (*A*) or ethidium bromide (*B*, *C* and *D*). On the right (*A´–D´*) are the same neurones after immunocytochemistry to show Na$_v$1.7-LI; these images were captured under interference contrast optics to increase clarity, but note that all image analysis was performed on bright field images. Sections of the dye-injected neurone in *A*, *B* and *C*, but not *D*, were through the nucleus. The C polymodal nociceptor in *A* shows intense labelling; both Aδ-fibre units (a D hair LTM unit in *B* and a nociceptive unit in *C*) show intermediate labelling. The muscle spindle afferent in *D* shows weak or absent labelling and, having no nucleus, does not indicate the full size of the cell. LTM, low threshold mechanoreceptor; HTM, high threshold mechanoreceptor.

Table 1. Correlations between Na$_v$1.7 relative intensity, cell size, CV and AP duration at base

		All				C				Aδ				Aδ/β				Noci				LTM			
	P	±	r²	n	P	±	r²	n	P	±	r²	n	P	±	r²	n	P	±	r²	n	P	±	r²	n
Intensity *vs.* size	****	−	0.35	66	n.s.			9	*	−	0.2	22	n.s.			36	**	−	0.25	29	**	−	0.25	34
Intensity *vs.* CV	****	−	0.31	66	n.s.			9	n.s.			22	n.s.			36	**	−	0.2	29	**	−	0.19	34
Intensity *vs.* APdB	****	+	0.53	57	**	+	0.89	7	n.s.			19	***	+	0.38	31	**	+	0.35	26	****	+	0.50	29
APdB *vs.* CV	****	−	0.36	57	n.s.			7	n.s.			19	n.s.			31	***	−	0.48	26	**	−	0.22	29
APdB *vs.* size	***	−	0.34	57	n.s.			7	n.s.			19	n.s.			31	*	−	0.23	26	*	−	0.20	29

For linear correlations shown in Fig. 5 (upper section of table), and for correlations between CV, cell size and AP duration (lower section of table), the following are shown: *P* values, whether correlations are positive (+) or negative (−), *r*² values and the number of units, *n*. In the first column, 'intensity' means relative intensity of Na$_v$1.7-LI, 'size' means cell cross-sectional area through the largest section in that cell and 'APdB' means AP duration at base. Units were included for CV and cell size regardless of membrane potential or AP height. Selection criteria for cells for including their AP durations are given in the text. The same data were subdivided according to CV (columns 3– 5) and then according to sensory properties (see legend to Fig. 5). The regression lines for nociceptive and LTM units were compared (GraphPad Prism software). In no case was there a significant difference in the slopes or elevations of these lines. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$; n.s., not significant.

C-PMs (not shown). Of the 15 units not included in Fig. 4, a C-cooling unit (CV 0.27 m s^{-1}) was intensely labelled (82 % relative intensity), four unresponsive C-fibre units were clearly positive (31–58 %), the Aδ-cooling unit and the moderate pressure unit were weakly positive (31 and 22 % respectively), five Aδ unresponsive units were all positive (20–51 %) but three unresponsive Aα/β-fibre units were negative. Thus in the unresponsive units, as well as the nociceptive and LTM units, those with the more slowly conducting fibres showed the highest relative intensities. These patterns, including the lack of staining in the muscle spindle afferents which tend to have CVs in the high Aα/β range, appeared to indicate that staining intensity may be greater in units with low CVs.

The relative intensity of Na$_v$1.7-LI was correlated negatively with cell size for all cells, Aδ neurones (weakly) and for both nociceptive and LTM neurones (Fig. 5*A* and

B, Table 1). The greater association of Na$_v$1.7 intensity with nociceptive units may therefore in part be secondary to its relationship with cell size, due to the smaller mean size of nociceptive units (see Fig. 5*B*).

The Na$_v$1.7 intensity was also negatively correlated with dorsal root CV for all units together (Fig. 5*C*), and for nociceptive and LTM neurones separately (Fig. 5*D*). The apparent difference in slopes of the nociceptive and LTM regression lines was not significant, and appears to be due to the greater preponderance of C-fibre units with nociceptive properties and fast conducting Aα/β-fibre units (> 12 m s^{-1}) with LTM properties. This view is supported by the almost perfect superimposition of the regression lines for nociceptive and LTM units (see Fig. 5*D* insert) when these extreme ends of the range were excluded and only units with CVs of 1.1–12 m s^{-1} were compared (see inset). In this range there were significant
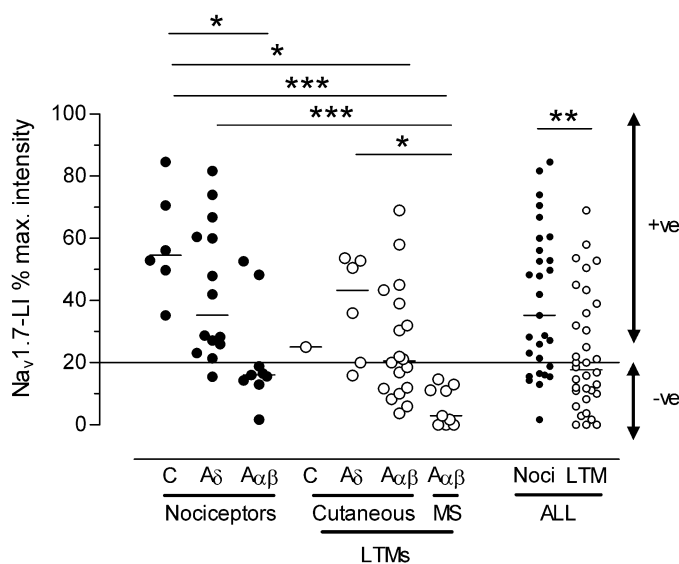
Figure 4. Relative intensities of Na$_v$1.7-LI in neurones defined by sensory properties and CV

A scattergraph showing the distribution of relative intensities of Na$_v$1.7-LI in the different groups of nociceptive and LTM (low threshold mechanoreceptive) neurones, and, in the last two columns, all nociceptive and all LTM neurones grouped together. ●, nociceptors; ○, LTMs. Cutaneous LTMs includes Aδ (D hair) units and Aα/β (G hair, field, slowly adapting and rapidly adapting) units; MS, mainly muscle spindle, proprioceptive, afferent units. Medians are shown. The Kruskall-Wallis test with Dunn's *post hoc* test was used to compare all subgroups except C LTMs (only one cell). The Mann-Whitney test was used to compare medians of all nociceptive and all LTM units. Medians that were significantly different are indicated with a line above the two groups and asterisks show the level of significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
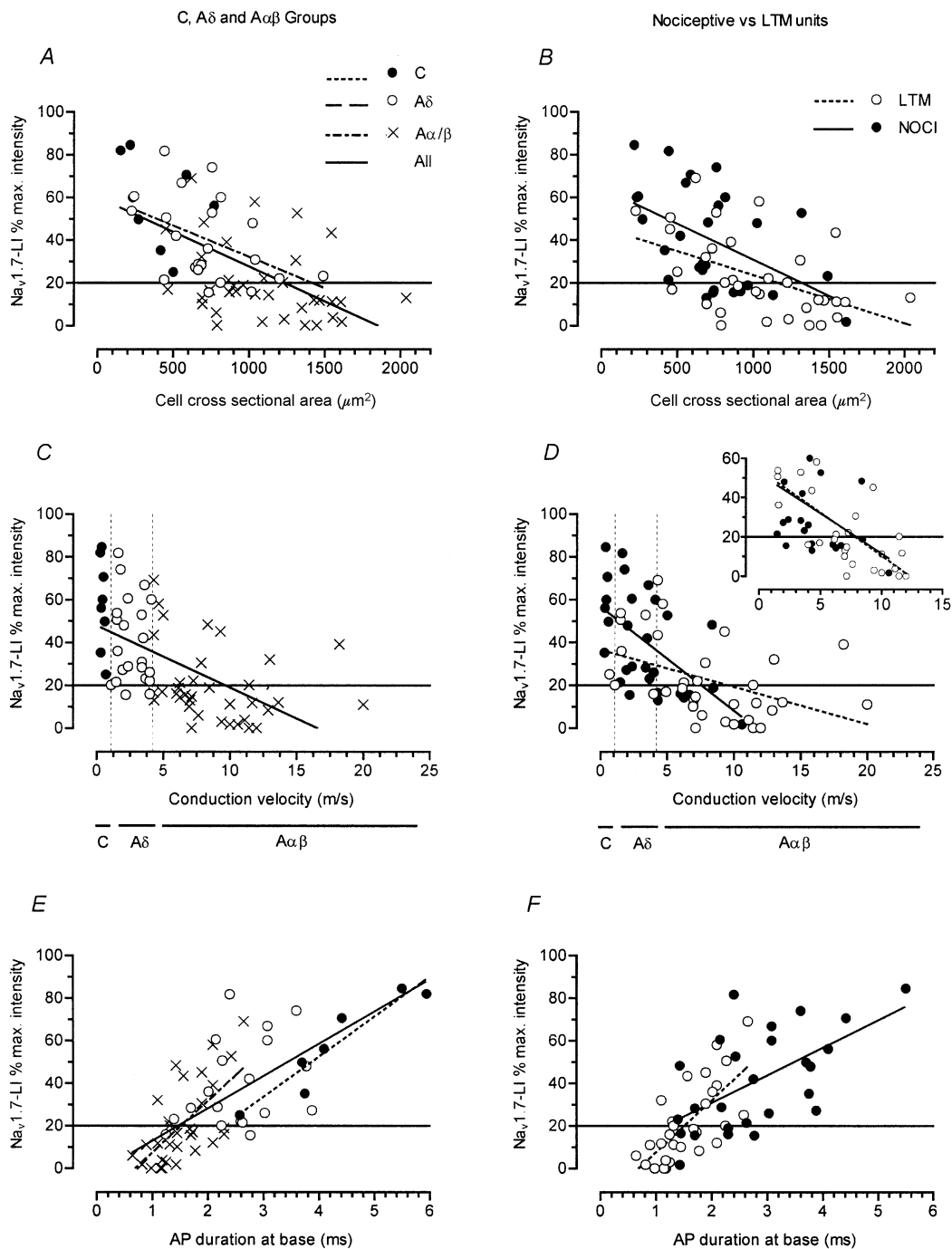
Figure 5. Graphs of Na$_v$1.7-LI intensity against neuronal properties

Neuronal properties plotted against Na$_v$1.7-LI intensity are cell size (the cross-sectional area of the largest section through the neurone; *A* and *B*); dorsal root CV (*C* and *D*); and AP duration (the duration at base of somatic APs evoked by electrical stimulation of the dorsal root; *E* and *F*). The horizontal line at 20% indicates the relative intensity above which cells are considered positive for Na$_v$1.7. The inset in *D* shows superimposition of the lines for nociceptive (NOCI) and low threshold mechanoreceptive (LTM) units when only units with CVs of 1.1–12 m s^{-1} were included (see text). On the left, units are subdivided into those with C- (●), Aδ- (○) or Aα/β-fibres (×); in these groups nociceptive and non-nociceptive units, and cooling-sensitive units were included. Unresponsive units were excluded. Linear regression analysis was carried out on each of these CV groups separately and on all units together, and regression lines are included only where there was a significant correlation. On the right, only nociceptive (●) and LTM units (○) are included; thus fewer units are plotted. Linear regression analysis was carried out on nociceptive and LTM units separately. The keys to symbols and lines may be found in *A* (for *A*, *C* and *E*) and *B* (for *B*, *D* and *F*). For significant linear correlations (*P* < 0.05), regression lines are shown here and the *P* and *r*^{2} values are shown in Table 1.

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correlations for both LTMs ($P < 0.001$, $r^2 = 0.48$) and nociceptors ($P < 0.05$, $r^2 = 0.2$). At least over this range, $Na_v1.7$-LI relative intensity therefore appears to be more closely associated with CV than with sensory receptive properties, although with relatively low r^2 values (Table 1). The greater association of $Na_v1.7$ intensity with nociceptive units may therefore, in part, be secondary to its relationship with CV, due to the overall lower CV of nociceptive units.

$Na_v1.7$-LI relative intensity showed significant positive correlations with AP duration at base for all neurones together, and for C and Aα/β units (Fig. 5E). The r^2 value for C-fibre neurones with identified receptive fields was high (Table 1); units included five nociceptors, a C-LTM and a C-cooling unit. The r^2 value was high even if only the nociceptive C-fibre units were included ($P = 0.02$, $r^2 = 0.86$, $n = 5$). However, the two C-unresponsive neurones that had membrane potentials of at least −40 mV did not fall near this line, having longer APs (7.8 and 10.8 ms) than the nociceptive units but only intermediate staining (49 and 31 % respectively). Figure 5F shows that the regression line for LTM units extended more to the lower left, and the line for nociceptive neurones continued more to the upper right of the graph. The tendency of nociceptive neurones to have longer AP durations than LTM neurones within the same CV group has previously been demonstrated (Ritter & Mendell, 1992; Djouhri *et al.* 1998). The pattern seen here appears to indicate that there is a stronger relationship of $Na_v1.7$-LI with AP duration than with sensory properties *per se*, and indeed that the more intense $Na_v1.7$-LI in nociceptive units appears to be secondary to their longer AP duration.

Of the above correlations, that between $Na_v1.7$-LI and AP duration at base was the strongest with the greatest r^2 value, and indeed this was the only variable that showed a significant correlation with $Na_v1.7$-LI within the C- and Aα/β-fibre groups. To examine the possibility that the correlations of $Na_v1.7$-LI with cell size and CV could have been secondary to the correlation with AP duration at base, linear regression analysis between AP duration and both cell size and CV was carried out on the same cells, see Table 1. Significant correlations were found between both pairs of variables for all cells together, and for all nociceptive and for all LTM neurones separately. Indeed, the correlations between intensity and AP duration at base, and between AP duration at base and CV may be to some extent responsible for the weaker correlation between intensity and CV.

DISCUSSION

$Na_v1.7$-LI was more intense in smaller neurones, and in neurones with slower CVs. It was not limited to nociceptive neurones, being present also in LTM neurones, although overall a higher proportion of

nociceptive than of LTM units were positive. $Na_v1.7$-LI intensity was more strongly correlated with somatic AP duration than with cell size or CV.

Preferential labelling of small DRG neurones was seen in the rat and guinea-pig with this well characterised antibody. It was also seen in the rat with an antibody raised to a different epitope on $Na_v1.7$, and with a further antibody that showed 'labelling of all cell types but with an increased intensity in small diameter cells' (Porreca *et al.* 1999).

Lack of membrane labelling

Na^+ channel immunostaining has been observed using sensitive immunofluorescence and confocal microscopy methods in sites of very high Na^+ channel density e.g. node of Ranvier or, with only weak confocal staining, in the postsynaptic membrane of skeletal muscle (Wood *et al.*1998; Caldwell *et al.* 2000; Caldwell & Levinson, unpublished). However, in the DRG somata the channel density is order/s of magnitude lower than in these sites (see Cummins & Waxman 1997, Schild & Kunze, 1997) and was not previously seen in the DRG soma membrane even using the most sensitive immunofluorescence techniques (e.g. see Toledo-Aral *et al.* 1997; Gould *et al.* 2000). Indeed, other studies have shown (Brismar & Gilly, 1987; Thornhill & Levinson, 1992; Ukomadu *et al.* 1992) that the cytoplasmic pool of Na^+ channel is usually much greater than that inserted into the soma membrane. It is therefore not surprising that it was not detectable in DRG cell membranes in this study, especially since a less sensitive non-fluorescence immunocytochemistry was employed. The pool of cytoplasmic protein in the DRG is presumably the source of the soma membrane channel protein, although much of it must be destined for the fibres whose surface areas are orders of magnitude greater than those of the soma. The correlations seen here between cytoplasmic $Na_v1.7$-LI and soma membrane properties appear to indicate that the amount of functional protein in the soma membrane is related to the amount of cytoplasmic channel protein despite being probably only a small proportion of the total soma channel protein.

$Na_v1.7$ protein *versus* mRNA

In adult rat DRGs, $Na_v1.7$ protein (Porreca *et al.* 1999; Gould *et al.* 2000) is more highly expressed in small than large neurones despite the presence of mRNA in neurones of all sizes (Black *et al.* 1996). In large neurones, the low protein levels may reflect (a) poor translational efficiency; (b) post-translational modification and/or (c) rapid transport from the somata. The lack of $Na_v1.7$-LI in large myelinated peripheral fibres (S.R. Levinson, unpublished observations) makes (c) unlikely. However, $Na_v1.7$-LI at nerve terminals in cultured E15 DRG neurones (Toledo-Aral *et al.* 1997) and clear $Na_v1.7$-LI in most unmyelinated (C-) fibres (S. R. Levinson, unpublished observations) does suggest transport of protein to certain fibres and

terminals. Present findings of somata with C-fibres having the highest Na$_v$1.7-LI support the view that staining intensity of the soma is directly related to that of the fibre.

Sensory receptive properties

In contrast to the pattern of expression of the TTX-R Na$^+$ channel α subunit Na$_v$1.9 which is expressed exclusively in nociceptive-type DRG neurones (Fang *et al.* 2002), Na$_v$1.7 is expressed in both nociceptive and LTM neurones. Thus a marker associated mainly with small neurones may (e.g. Na$_v$1.9) or may not (e.g. Na$_v$1.7) be exclusively associated with nociceptive neurones. Nonetheless, even Na$_v$1.7 was in a higher proportion of nociceptive than LTM units, with higher median staining intensity in nociceptive units. Indeed, we would expect the proportion of nociceptive units that are positive would have been even greater if small neurones with slow CVs had not, as usual in this type of study, been under-represented.

Action potential duration

The clear relationship between Na$_v$1.7 and AP duration especially in C-fibre neurones indicates a relationship between cytoplasmic Na$_v$1.7 protein levels and levels of functional channel protein in the soma membrane. There is a similarity in the pattern of both Na$_v$1.7-LI intensity and AP duration at base in relation to sensory properties and CV, which is also consistent with a functional link between Na$_v$1.7-LI and AP duration. Both are graded with CV, being most extreme (i.e. most intense Na$_v$1.7-LI and longest AP durations) in nociceptive units with slow CVs, and less extreme but with a similar graded pattern with CV in LTM neurones. This pattern of nociceptive properties was classed as pattern A (Lawson, 2002).

Possible explanations for slower AP kinetics being linked with more Na$_v$1.7 protein include (a) the possibility of slower activation/inactivation kinetics for Na$_v$1.7 than for the other TTXS subunits that dominate the Na$^+$ channel complement in larger DRG neurones (Cummins *et al.* 1998); (b) co-expression with other subunits that results in slow AP kinetics (e.g. Na$_v$1.8); (c) influence of Na$_v$1.7 on the kinetics of other Na$^+$ channel subunits. The probable complexities of interaction between channel subtypes make it impossible to speculate further, although the data do support a role for Na$_v$1.7 in generation of the AP inward current.

Conduction velocity

The stronger correlation of somatic Na$_v$1.7-LI intensity with somatic AP duration than with fibre CV, taken with the correlation between soma AP duration and fibre CV, may indicate that the relationship between Na$_v$1.7-LI and CV is secondary to that with soma AP duration. This would make sense if functional channel subtypes in soma and fibre membranes are similar, since slower AP rise times in the fibre would result in slower conduction along the fibre.

Clinical relevance

Na$_v$1.7 may not, at first sight, appear a good choice as a target for a Na$^+$ channel-directed novel analgesic drug due to its expression in LTM neurones and in sympathetic neurones which may lead to unwanted side effects. Nonetheless, the present data may indicate that targeting this subunit could influence nociceptive more than non-nociceptive DRG neurones. More information is needed about its role especially in models of chronic pain before it can usefully be assessed as a possible therapeutic target.

In summary, although Na$_v$1.7 is not limited to nociceptive neurones, it is higher in DRG neurones with broader somatic APs that have slower CVs and smaller somata. These properties are associated with nociceptive neurones, which may account for the overall greater expression of Na$_v$1.7 in nociceptive than LTM neurones. Targeted manipulation of Na$_v$1.7 expression followed by examination of membrane function in identified neurones would help to determine the contribution of Na$_v$1.7 to DRG membrane properties.

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