The Presence and Role of the Tetrodotoxin-Resistant Sodium Channel $Na_v1.9$ (NaN) in Nociceptive Primary Afferent Neurons

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This is the first examination of sensory receptive properties and associated electrophysiological properties *in vivo* of dorsal root ganglion (DRG) neurons that express the TTX-resistant sodium channel $Na_v1.9$ (NaN). Intracellular recordings in lumbar DRGs in Wistar rats enabled units with dorsal root C-, Aδ-, or Aα/β-fibers to be classified as nociceptive, low-threshold mechanoreceptive (LTM), or unresponsive. Intracellular dye injection enabled subsequent immunocytochemistry for $Na_v1.9$-like immunoreactivity ($Na_v1.9$-LI).

$Na_v1.9$-LI was expressed selectively in nociceptive-type (C- and A-fiber nociceptive and C-unresponsive) units. Of the nociceptive units, 64, 54, and 31% of C-, Aδ-, and Aα/β-fiber units, respectively, were positive for $Na_v1.9$-LI. C-unresponsive units were included in the nociceptive-type group on the basis of their nociceptor-like membrane properties; 91% were positive. $Na_v1.9$-LI was undetectable in Aδ- or Aα/β-fiber LTM units and in one C-LTM unit. $Na_v1.9$-LI intensity was correlated negatively with soma size and conduction velocity in nociceptive units and with conduction velocity in C-fiber units. There was a positive correlation with action potential rise time in nociceptive-type units with membrane potentials equal to or more negative than –50 mV. The data provide direct evidence that $Na_v1.9$ is expressed selectively in (but not in all) C- and A-fiber nociceptive-type units and suggest that $Na_v1.9$ contributes to membrane properties that are typical of nociceptive neurons.

Key words: action potential; conduction velocity; DRG; $Na_v1.9$ (NaN); pain; sodium channel

Voltage-gated Na^+ channels are important in regulating neuronal excitability and in the initiation and propagation of action potentials. On the basis of sensitivity to tetrodotoxin (TTX) and kinetic properties, Na^+ currents in dorsal root ganglion (DRG) neurons can be classed as fast TTX-sensitive (TTX-S), slow TTX-resistant (TTX-R) with a high-activation threshold, and persistent TTX-R with much lower activation thresholds (Waxman, 1999). Most primary sensory neurons express TTX-S current (Kostyuk et al., 1981; Caffrey et al., 1992; Catterall, 1992; Roy and Narahashi, 1992), and it is thought that several α-subunits are involved in generating this current (Akopian et al., 1996; Black et al., 1996; Sangameswaran et al., 1997). However, the different TTX-R currents in small/medium-sized DRG neurons (Roy and Narahashi, 1992; Elliott and Elliott, 1993; Arbuckle and Docherty, 1995; Rush et al., 1998) are thought to be mediated by two sensory neuron-specific Na^+ channels. These are known according to the new standardized nomenclature (Goldin et al., 2000) as $Na_v1.8$ (SNS/PN3) (Akopian et al., 1996; Sangameswaran et al., 1996) and $Na_V1.9$ (NaN/SNS2) (Dib-Hajj et al., 1998; Tate et al., 1998; Fjell et al., 2000). Because of their preferential expression in small-diameter DRG neurons (Dib-Hajj et al., 1998; Tate et al., 1998; Amaya et al., 2000; Sleeper et al., 2000) and the presence of TTX-R currents in neurons with broad/inflected action potentials (Gallego, 1983; Waddell and Lawson, 1990), the channels encoding TTX-R currents are of particular interest because they may make important contributions to the membrane properties of nociceptive primary afferent neurons (Gold, 1999; McCleskey and Gold, 1999). However, relatively little is known of the possible contribution of $Na_v1.9$ to nociceptive transmission.

$Na_v1.9$ (NaN) initially was cloned and sequenced by Dib-Hajj et al. (1998) and subsequently by Tate et al. (1998), who called it SNS2. On the basis of its sequence $Na_v1.9$ was predicted to encode a TTX-R Na^+ channel (Dib-Hajj et al., 1998). However, $Na_v1.9$ channels have very different properties from TTX-R $Na_v1.8$ channels. The hyperpolarized voltage dependence of activation and the persistent (noninactivating) nature of $Na_v1.9$ current (Cummins et al., 1999) are thought to depolarize the membrane in small DRG neurons (Herzog et al., 2001).

$Na_v1.9$ is colocalized with $Na_v1.8$ in small-diameter DRG neurons (Tate et al., 1998; Amaya et al., 2000), but unlike $Na_v1.8$, which is expressed in small- and medium-sized DRG neurons, $Na_v1.9$ was reported by some investigators to be only in small-diameter (10–25 μm) DRG neurons (Tate et al., 1998; Amaya et al., 2000) (but see Discussion). Although slowly conducting neurons tend to be small (Harper and Lawson, 1985), there is considerable overlap in cell size among DRG neurons with C-, Aδ-, and Aα/β-fibers (Harper and Lawson, 1985). In addition, there are nociceptive neurons with fibers that conduct in each of these conduction velocity ranges (for review, see Lawson, 2002). Thus

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the common assumption that small/medium-sized DRG neurons are nociceptive and that markers specific for small DRG neurons are associated with nociceptors may be misleading. Such markers may be related to nociceptive function, to slow conduction velocity, or to some other functional property related to cell size. It is important to determine which Na^+ channel subtypes are expressed specifically in nociceptive neurons and are responsible for their membrane properties. The aim of the present study was, therefore, to ascertain directly the sensory receptive properties and conduction velocities of DRG neurons that express $Na_v1.9$ protein and, further, to examine whether membrane properties of neurons expressing $Na_v1.9$ are related to the intensity of the somatic $Na_v1.9$-like immunoreactivity ($Na_v1.9$-LI).

MATERIALS AND METHODS

Experiments were performed on young female Wistar rats (weight 150–180 gm) deeply anesthetized with an initial dose of 60 mg/kg intraperitoneally of sodium pentobarbitone. A tracheotomy was performed to allow artificial ventilation and end-tidal CO_2 monitoring. The anesthetic dose produced deep anesthesia with areflexia (i.e., total absence of limb withdrawal reflex). The left carotid artery was cannulated to enable regular intra-arterial (i.a.) injections of additional doses of the anesthetic (10 mg/kg) that were required to maintain this deep level of anesthesia. Blood pressure also was monitored throughout in one-half of the experiments. The animal core temperature was maintained throughout at 36°C (± 0.5), and the temperature in the paraffin pool was maintained at 28–32°C. Experimental procedures complied throughout with Home Office Guidelines. Full details of the animal preparation were as reported previously in guinea pig (Lawson et al., 1997; Djouhri and Lawson, 1999).

For stability during electrophysiological recording, just before recording the animals were given a muscle relaxant, pancuronium (0.5 mg/kg, i.a.), always accompanied by an additional dose (10 mg/kg, i.a.) of the anesthetic. These same doses of muscle relaxant and anesthetic (always given together) were administered at regular intervals (approximately hourly). These anesthetic doses were the same as those that induced deep anesthesia during the period (2–3 hr) of animal preparation. In the later experiments the blood pressure measurements indicated that, by our using these same doses of muscle relaxant and anesthetic, the blood pressure was stable, showing no indication of any reduction in the depth of anesthesia at any stage in the experiment.

Intracellular recordings. Glass microelectrodes filled with fluorescent dye were used for intracellular recordings from DRG neuronal somata. The fluorescent dyes were Lucifer yellow (LY) in 0.1 M LiCl (360 ± 154 MΩ, mean ± SD), ethidium bromide (EB) in 1 M KCl (127 ± 89 MΩ), or occasionally cascade blue (CB) as a 3% solution in 0.1 M LiCl (455 ± 120 MΩ). The microelectrode was advanced in 1 μm steps until a membrane potential was seen and an action potential could be evoked by dorsal root stimulation with single rectangular pulses (0.03 msec duration for A-fiber units or 0.3 msec for C-fiber units). Then the stimulus intensity was adjusted to usually twice threshold for A-fiber units and between one and two times threshold for C-fiber units. The somatic action potentials were recorded on-line with a CED (Cambridge Electronic Design, Cambridge, UK) 1401 plus interface and the SIGAV program from CED and subsequently were analyzed off-line with the Spike II program (CED).

Action potential variables also were measured for each unit as described previously [Djouhri and Lawson (1999), their Fig. 1]. These were the action potential duration at base, action potential rise time (time from inception of the action potential to the peak), and action potential fall time (time from the peak to the point at which the potential crosses or reaches the resting membrane potential).

Conduction velocity. The conduction velocity of each unit was estimated from (1) the latency between the dorsal root stimulus and the onset of the evoked somatic action potential and (2) 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 neuron in the DRG and was between 4.5 and 13 mm. Utilization time was not taken into account. Note that dorsal root fibers in rats of this age tend to conduct somewhat more slowly than peripheral nerve fibers of the same DRG neurons, by an average of 14% in A-fiber neurons and 28% in C-fiber neurons (Waddell et al., 1989).

Compound action potentials recorded from dorsal roots using the methods described by Djouhri and Lawson (2001a) provided the basis for the subdivision of neurons into C-, Aδ-, and Aα/β-fiber neurons as follows. The borderline between Aδ and Aα/β waves was determined in three L5 dorsal roots and one L6 dorsal root from different animals, keeping sex, age/weight, and temperature the same as in this experimental series. The mean value (±SD) for the borderline between Aδ and Aα/β waves was 6.5 ± 0.32 m/sec; the values for L6 and L5 were similar. Two C waves were seen ($n = 2$) (see also Djouhri and Lawson, 2001a). The fastest component of the slower C wave displayed a conduction velocity of 0.7–0.8 m/sec, whereas the fastest component of the faster wave, which we shall designate as a C/Aδ wave, conducted at 1.4–1.5 m/sec. Neurons were classified according to their dorsal root conduction velocity as C (<0.8 m/sec), C/Aδ (0.8–1.4 m/sec), Aδ (1.4–6.5 m/sec), and Aα/β (>6.5 m/sec).

Sensory receptive properties. The sensory receptive properties of DRG neurons were examined with hand-held stimulators; they were identified and classified as described previously (Lawson et al., 1997; Djouhri et al., 1998). Briefly, units were tested for their responses to low-intensity (non-noxious) mechanical stimuli by lightly brushing the limb fur with a soft brush, skin contact, very slow movement and light pressure with blunt objects, light tap, tuning forks vibrating at 100 or 250 Hz, and pressure with calibrated von Frey hairs. Noxious mechanical stimuli were applied with a needle, pinch of superficial tissue with fine forceps, or pinch of skin and deeper tissues with coarse flat or coarse-toothed forceps as described in previous studies (Lawson et al., 1997; Djouhri et al., 1998). Noxious heat was applied to the leg with hot water at >50°C, and cooling of the skin was with a very brief spray of ethyl chloride or ice applied to the skin.

Units responding to non-noxious mechanical stimuli were categorized as low-threshold mechanoreceptive (LTM) units. Those with Aδ-fibers that were extremely sensitive to the slow movement of hairs and to cooling stimuli with a prolonged discharge were classified as Aδ LTM down hair (D hair) units. Most Aα/β LTM units in this study were cutaneous, with superficial or dermal receptive fields (Lawson et al., 1997), but a group of Aα/β-fiber LTM units with deeper receptive fields that often showed ongoing discharges, that responded to light pressure against muscle tissue, and that followed a vibration of 100 or 250 Hz applied with a tuning fork were classified as muscle spindle (MS) afferent units. The MS group may have included Golgi tendon organ afferents.

Units not responding to the low-intensity stimuli were tested with noxious mechanical and thermal stimuli. A-fiber units responding to the former, but not to the low-intensity non-noxious mechanical stimuli, were classified as high-threshold mechanoreceptive (A-HTM) units; a few units also responding to cooling were classified as A-mechano-cooling (A-MC). The term "A-fiber nociceptive units" as used in this paper includes HTM and MC units; no A-mechanoheat units were encountered in these experiments.

The term "C-nociceptive units" in this paper includes C-polymodal units with receptive fields in the superficial cutaneous tissue that responded vigorously to both noxious heat and noxious mechanical stimuli, C-high-threshold mechanoheat (C-MH) units that responded to these stimuli but had receptive fields in the deep cutaneous tissues, and C-high-threshold mechanoreceptive (C-HTM) units (Lawson et al., 1997). C-HTM units were cutaneous units that required strong mechanical stimulation but lacked prompt responses to noxious heat or were units with deep receptive fields that responded to strong mechanical stimulation; the latter were not tested with thermal noxious stimuli. Specific heat and cooling units were not found in this study.

C-fiber low-threshold mechanoreceptive (C-LTM) units (C-mechanoreceptors) were those units that responded preferentially to very gentle contact moving across the skin at <1 mm/sec and sometimes to cooling as previously reported in several species (Light and Perl, 1993).

Unresponsive units with A- or C-fibers were those for which no receptive field was found despite an extensive search with the non-noxious and noxious mechanical and thermal stimuli described above. Unresponsive neurons have been described in several species, and it has been suggested that these units may be the so-called "silent nociceptors" (Handwerker et al., 1991; Meyer et al., 1991; Gee et al., 1996; Djouhri and Lawson, 1999). Measures typical of nociceptive as compared with LTM neurons (long action potential and afterhyperpolarization durations and large action potential overshoots; values not shown) were very similar in the C-unresponsive units and C-nociceptive neurons both in this study and in the guinea pig (Djouhri et al., 1998; Djouhri and Lawson, 2001b). These values were very different from those of the

C-LTM unit in this study and of those in the guinea pig (Djouhri et al., 1998), which had much shorter action potential and afterhyperpolarization durations and very small action potential overshoots. This suggests that the C-unresponsive units were probably either silent nociceptors with very high thresholds or nociceptors with inaccessible receptive fields. The term "nociceptive-type" neuron is used from this point on to include C- and A-fiber nociceptive neurons and C-unresponsive neurons. In contrast, the Aα/β-fiber unresponsive neurons in the present study had action potential and afterhyperpolarization durations much closer to those of the Aα/β-LTM units than those of Aα/β nociceptive units both in this study and in the study of Djouhri et al. (1998), indicating that these were more likely to be LTM units with inaccessible receptive fields, perhaps on the dorsal surface of the foot that was glued down to improve stability.

Neuronal labeling. Once each unit was characterized as described above, dye was ejected into the soma electrophoretically from the electrode by rectangular current pulses (usually 1 nA with a maximum of 1.3 nA for 500 msec at 1 Hz) for periods of up to 10–15 min for A-fiber neurons and 6–10 min for C-fiber neurons. Membrane potential was monitored every 30 sec throughout the injection time. The currents were negative for LY and CB and positive for EB. In L5 DRGs, which were 2 mm long, three neurons were labeled by LY (two at opposite ends of the ganglion and one in the middle). A further two neurons were injected with EB between the first and second and between the second and third LY-labeled neurons, respectively. Finally, neurons were labeled for CB at locations lateral to the tracks made with LY electrodes. Overall, of the cells included in this study, 55 cells were labeled with LY, 38 with EB, and 9 with CB. The problems of identifying the locations of dye-injected cells have been discussed previously (Lawson et al., 1997), and all precautions outlined in that report were taken and are now routine procedure.

At the end of the experiment the animal was perfused terminally under deep anesthesia through the heart with 0.9% saline, followed by Zamboni's fixative (Stefanini et al., 1967). The DRGs were left overnight in 30% sucrose in 0.1 M phosphate buffer at 4°C after they had been postfixed for ~1 hr in the same fixative. Serial 7 μm cryostat sections then 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 labeled neurons were recorded with camera lucida drawings and images captured on a high-resolution CCD camera (Optronix Dei-470). For each dye-labeled neuron the cross-sectional area of the largest section through the cell was used as a measure of cell size.

Immunocytochemistry. Before immunocytochemistry, endogenous peroxidase was blocked with 2% H_2O_2. Then endogenous biotin-like activity was blocked by using an avidin–biotin (Vector Laboratories, Peterborough, UK) kit, and the sections were incubated for 1 hr with 10% normal goat serum in PBS. Avidin–biotin complex immunocytochemistry was performed with an ABC kit (Vector Laboratories). Briefly, sections were incubated for 2 d at 4°C in primary antibody against the $Na_v1.9$ α-subunit (1.7×10^{-3} μg/ml) in 0.3% Triton X-100 in Tris buffer with 1% normal goat serum. They were incubated for 30 min at room temperature with biotinylated secondary antibody (anti-rabbit Ig, 1:200; Vector Laboratories). DAB was used to form a colored reaction product. The sections were dehydrated and the slides coverslipped. The anti-$Na_v1.9$ antibody was well characterized (Fjell et al., 2000), and its specificity in adult rat DRG tissue has been confirmed via Western blots (Tyrrell et al., 2001). No staining was seen when the procedure described above was used but PBS was used instead of the primary antibody.

A semiquantitative method was used to assess the relative intensity of the immunostaining for $Na_v1.9$. The relative intensity was the relative absorbance of light by the reaction product in the cytoplasm of each dye-labeled cell, relative to that of the cytoplasm of other cells in the same section. When we used a macro program running in NIH Image (L. Djouhrin, R. Newton, S. R. Levinson, and S. N. Lawson, unpublished observations), the mean absorbance of the darkest 10% of the pixels in the cytoplasm of the dye-injected neuron (c) was compared with the mean absorbance of three clearly negative neurons in the vicinity of the labeled cell (a; taken as 0% intensity) and with the mean absorbance of the three most intensely stained profiles in the section (b; taken as 100% intensity) as follows: relative intensity of dye-labeled cell as a percentage $= (c - a)/(b - a)$.

This method enables labeling that is uneven, e.g., punctate or globular, to be distinguished clearly above background levels. To validate this method, all dye-injected neurons stained to show $NA_v1.9$-LI were also scored subjectively by agreement between two observers as negative for $Na_v1.9$-LI (clearly unlabeled), positive (clearly labeled), and borderline positive cells were scored subjectively on a scale of 1 (weak positive) to 5 (as dark as the most intensely stained profile in the section). The subjective measures were repeatable between different observers and were highly positively correlated with relative intensity as calculated by using Image analysis ($r^2 = 0.96$; $p < 0.0001$; $n = 102$).

Neurons with relative absorbance ≥20% were judged consistently as being clearly positive (≥1 on subjective rating), and those with values of <20% were judged consistently as being negative (<1) by all viewers. The term "positive" therefore is used to refer to neurons with relative absorbances ≥20%, and the term "negative" refers to units with relative absorbances <20%. We cannot exclude the possibility that some cells with absorbances <20% and classified as negative may have had low levels of $Na_v1.9$ protein. This approach can be classed only as semiquantitative. Nonetheless, the use of objective comparisons of the cell staining with the full range of staining within that section removes much of the variability caused by differences between different immunocytochemical reactions.

For the analysis of the action potential characteristics, data were included only if neurons had membrane potentials more negative than −50 mV, if the temperature at the DRG was 28–32°C and the somatic action potential was overshooting. Units that had a marked inflection on the rising phase of the action potential were excluded ($n = 2$, both C-fiber units) because these were atypical.

RESULTS

A total of 102 DRG neurons with identified sensory receptive properties was labeled with a fluorescent dye, successfully recovered from histology, and examined for $Na_v1.9$-like-immunoreactivity (LI). These included 40 nociceptive units (11 C-, 13 Aδ-, and 16 Aα/β-fiber), 16 unresponsive units (11 C- and 5 Aα/β-fiber), and 46 LTM units (2 C-, 5 Aδ-, and 39 Aα/β-fiber). The staining varied from most intense in small C-fiber neurons to least intense in the large Aα/β-fiber neurons.

The proportions of units with each type of sensory property are not necessarily indicative of proportions in the DRG, because certain influences may bias the collection of these data. These include the greater difficulty of making stable recordings from units with small somata and of identifying receptive fields of units with deep and/or high-threshold receptive fields; the longest searches were for receptive properties of units that proved to be unresponsive. The longer the search for the receptive field site or properties, the greater the chance of losing the unit before dye injection.

$Na_v1.9$-LI-positive and $Na_v1.9$-LI-negative neurons

Examples of $Na_v1.9$-LI in neurons with identified sensory properties are shown in Figure 1, including an intensely labeled C-fiber nociceptive unit, a moderately labeled C-unresponsive unit, and examples of negative Aδ- and Aα/β-fiber units.

$Na_v1.9$-LI intensity

Figure 2 shows the distribution of $Na_v1.9$-LI relative intensity of neurons in relation to their sensory properties; 20% relative intensity (Fig. 2, *dotted line*) is used as the borderline between positive and negative units (see Materials and Methods).

Most C-fiber nociceptive-type neurons (7 of 11, 64% of nociceptive; 10 of 11, 91% of unresponsive) were clearly $Na_v1.9$-positive. The single typical C-fiber LTM unit that we encountered was negative for $Na_v1.9$. An atypical C-fiber unit with properties intermediate between LTM and nociceptive units was positive.

Of the A-fiber neurons, approximately one-half (7 of 13) of the Aδ-nociceptive neurons were clearly $Na_v1.9$-LI-positive (intensity, 29–68%). The remaining Aδ-nociceptive neurons and all five Aδ-LTM (D hair) neurons were $Na_v1.9$-LI-negative. Of the

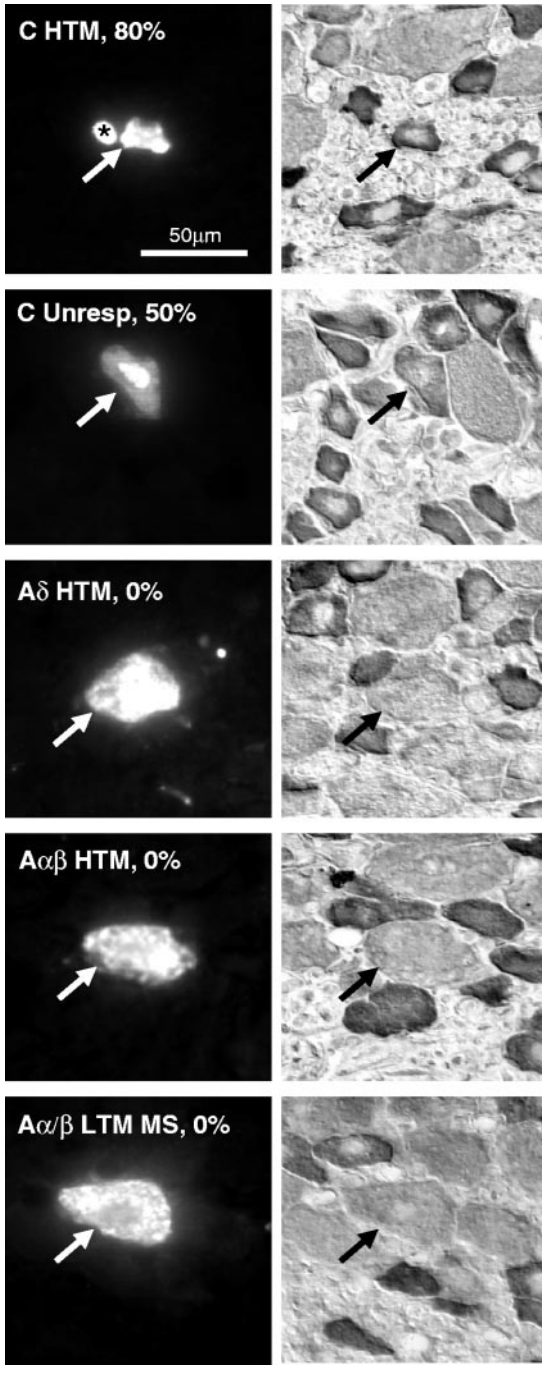

Figure 1. Photomicrograph examples of representative $Na_v1.9$-LI-positive C-fiber nociceptive and C-fiber unresponsive units and negative A-fiber nociceptive and LTM units. Units injected with a fluorescent dye are marked with an *arrow* on the *left*; their sensory receptive type and $Na_v1.9$-LI relative intensity as a percentage are shown. The same neurons stained for $Na_v1.9$-LI are shown on the *right*. In all units the injected fluorescent dye was ethidium bromide except for the C-unresponsive unit, which was injected with cascade blue. *Unresp*, Unresponsive; *HTM*, high-threshold mechanoreceptive; *LTM*, low-threshold mechanoreceptive; *MS*, muscle spindle. The *asterisk* in the *top left* image indicates a dust particle. Scale bar, 50 μm (applies to all images).

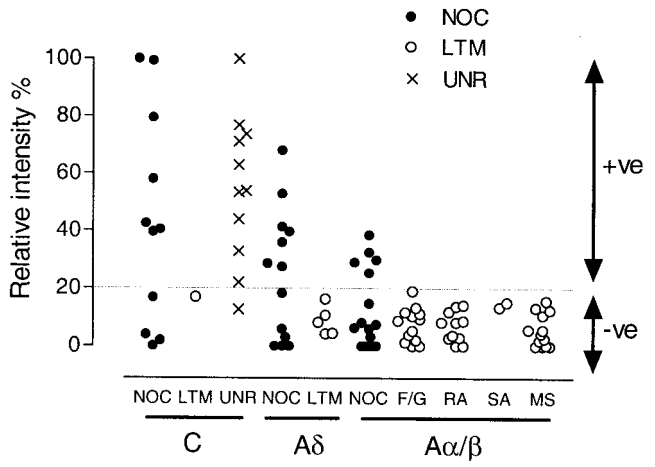

Figure 2. Scattergraph of distributions of $Na_v1.9$-LI relative intensity (*y*-axis) in neurons subdivided by sensory receptive type (*x*-axis) and into C-, Aδ-, and Aα/β-fiber groups. The *faint line* on the *y*-axis indicates the 20% borderline between $Na_v1.9$-LI-positive and $Na_v1.9$-LI-negative neurons. *NOC*, Nociceptive neurons; *LTM*, low-threshold mechanoreceptive neurons; *UNR*, unresponsive neurons; *F/G*, field or guard hair neurons; *MS*, muscle spindle neurons; *RA*, rapidly adapting LTM cutaneous afferent neurons; *SA*, slowly adapting cutaneous afferent neurons; *−ve*, negative; *+ve*, positive

faster-conducting Aα/β-fiber neurons, only ~31% (5 of 16) of the nociceptive neurons were $Na_v1.9$-LI-positive, and none of these was strongly positive (29–39%). The five unresponsive Aα/β-fiber units (conduction velocity, 11.43–21.54 m/sec) were all $Na_v1.9$-LI-negative (0–3%; data not shown). Because they were probably LTM units with inaccessible receptive fields (see Materials and Methods), the A-fiber unresponsive units were not considered further as a separate group.

Thus all of the LTMs that were examined were $Na_v1.9$-LI-negative (<20%; see also Fig. 2). These negative neurons included a C-LTM neuron, D hair units, field or guard hair (F/G), muscle spindle (MS) neurons, rapidly adapting (RA) neurons, and slowly adapting (SA) neurons. Conversely, every $Na_v1.9$-positive neuron was nociceptive-type (nociceptive or C-unresponsive), as seen in Figure 2.

$Na_v1.9$-LI relative intensity and conduction velocities

Because $Na_v1.9$ was expressed in C and Aδ, but rarely in larger fibers (Fjell et al., 2000; Liu et al., 2001), we examined the correlation between $Na_v1.9$-LI relative intensity of DRG somata and the conduction velocities of their dorsal root fibers; LTMs were excluded because they were all negative. There was a significant negative correlation in all units and in nociceptive units (Fig. 3*A*). No correlation was found between $Na_v1.9$-LI relative intensity and conduction velocity for Aδ- or Aα/β-fiber nociceptive units (data not shown).

C-fiber units

In contrast, when C-fiber units with conduction velocities ≤0.8 m/sec were examined (equivalent to the slow C wave of the compound action potential), a significant negative correlation between $Na_v1.9$-LI relative intensity and conduction velocity was found for C-nociceptive units, C-unresponsive units, and for all C-fiber units together (Fig. 3*B*).

$Na_v1.9$-LI intensity and soma cross-sectional area

Several studies describe $Na_v1.9$ as restricted entirely to small DRG neurons, whereas other studies indicate that it is also present in a small proportion of medium/large-sized cells (see Discussion). We examined the relationship of $Na_v1.9$-LI relative intensity to DRG neuronal soma size in 63 DRG neurons in

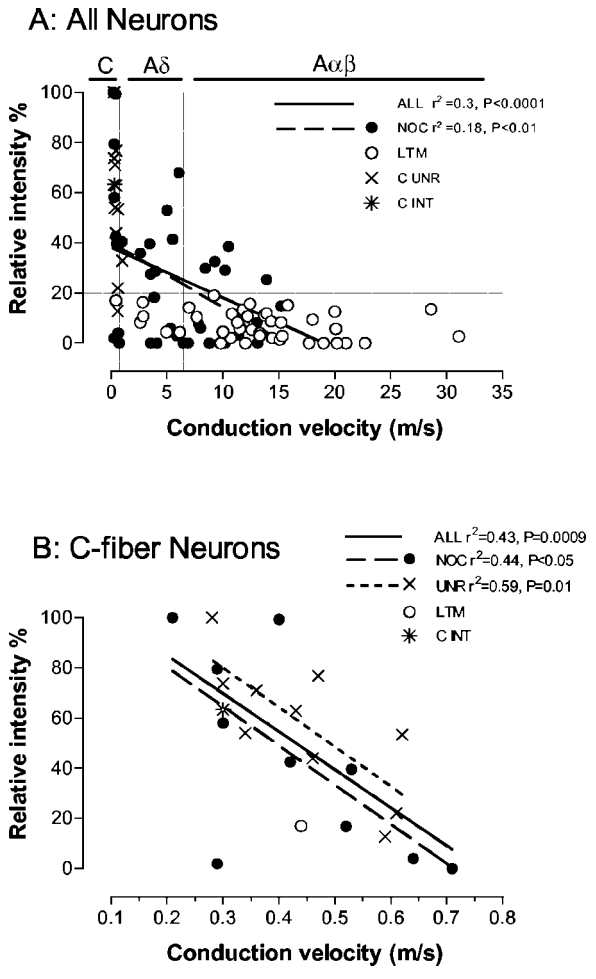

Figure 3. Relationships between soma $Na_v1.9$-LI relative intensity and neuronal dorsal root conduction velocities in all neurons (*A*) and in C-fiber neurons (*B*). Linear regression analysis was performed for all neurons: for nociceptive neurons in *A* and for all C-fiber units and separately for C-fiber nociceptive and C-fiber unresponsive neurons in *B*. Regression lines, *p*, and r^2 values are given where the correlation was significant ($p < 0.05$). The *line* at 20% from the *y*-axis shows the borderline between $Na_v1.9$-LI-positive and $Na_v1.9$-LI-negative neurons. The *vertical lines* from the *x*-axis (*A*) show the borderlines between slow C- and C/Aδ-fibers (0.8 m/sec), between the C/Aδ- and Aδ-fibers (1.5 m/sec), and between Aδ- and Aα/β-fibers (6.5 m/sec). Note that r^2 values are relatively high for $Na_v1.9$-LI intensity versus conduction velocity for C-fiber neurons. Abbreviations are as in Figure 2; *INT*, an intermediate C-cell with properties between those of an LTM and a nociceptive unit (see Results).

which the largest section through the cell was available; it was not available in the other 39 neurons.

In addition, for comparison with the above experimental neurons, the cross-sectional areas of all neuronal profiles containing a nucleus in one section taken every 140 μm through an L4 DRG from a 150 gm female rat are plotted (crosshatched histogram) in Figure 4*A*. Superimposed in gray and in black are all of the cells with relative intensity ≥20 and ≥50% of maximum, respectively. These percentages were calculated as follows: the minimum (0%) is the average overall pixel intensity in the cytoplasm for the 10 least intensely labeled neurons, and the maximum (100%) is the average for the 10 most intensely labeled cells; each cell is scaled between these two values as described previously. By comparison of this with distributions of two L5 DRGs from rats used in these experiments, the small, medium, and large cell boundaries were set as follows: neurons within the small cell peak are called "small" (up to 400 μm^2), and those above 800 μm^2 area are called "large" because this includes only the right-hand side of the large light cell distribution (Lawson et al., 1984); between these (400–800 μm^2) the neurons are "medium" sized (Fig. 4*A*). (Note that these values relate to size distributions for DRG neurons in the present study but may vary with changes in size distributions according to species, age, tissue processing etc.)

Figure 4 shows that most small neurons in the whole DRG (Fig. 4*A*) and in the group of impaled dye-injected neurons (Fig. 4*B*) are positive, and the strongest immunoreactivity is in the smallest neurons in the whole DRG (Fig. 4*C*) and in the dye-injected group (Fig. 4*D*). A few medium-sized neurons showed strong immunoreactivity, and even some large neurons also show weak-to-moderate immunoreactivity. The similarity in the relationship between cell size and intensity in uninjected (Fig. 4*C*) and dye-injected neurons (Fig. 4*D*) indicates that the dye and dye injection regime had little or no effect on the relative intensity of the $Na_v1.9$-LI.

The size distributions of $Na_v1.9$-LI-positive neurons with C-, Aδ-, and Aα/β-fibers (Fig. 4*B*) for which size measurements were available show that most $Na_v1.9$-LI-positive C-fiber neurons (13 of 15) were small (<400 μm^2) and had intense staining (≥50% relative intensity). Most Aδ-fiber neurons were medium-sized; positive cells were stained less intensely (also see Figs. 2, 4*D*) than the C-fiber neurons; the Aα/β-fiber neurons were medium and large, with a few positive cells (not intensely stained; 20–45% relative intensity) within the large cell size range.

A significant negative correlation between cytoplasmic $Na_v1.9$-LI intensity and cell size was found for nociceptive and for all neurons. LTM neurons were all negative (Fig. 4*D*).

$Na_v1.9$-LI intensity and action potential variables

Because Na^+ channels are essential in regulating action potential configuration in excitable cells, the correlation between $Na_v1.9$-LI intensity and action potential variables (action potential duration at base, action potential rise time, action potential fall time) was examined in neurons with membrane potentials equal to, or more negative than, –50 mV. Correlations between these variables and $Na_v1.9$-LI intensity were examined in those groups of neurons shown to express $Na_v1.9$. Linear regression analysis was performed on all such neurons, on C-nociceptive-type (nociceptive and C-unresponsive neurons together), and on Aδ- and Aα/β-fiber nociceptive neurons separately. Overall, more intense $Na_v1.9$-LI was seen in neurons with longer action potential durations. There were significant positive correlations between action potential rise time, action potential fall time, and action potential duration at base (Fig. 5). Action potential rise time showed the clearest correlation (highest r^2 value) (Fig. 5*B*). In addition, action potential rise time, but not fall time, showed a significant correlation with $Na_v1.9$-LI in C-nociceptive-type neurons (Fig. 5*B,C*). The lack of correlation in C-nociceptive-type units with action potential fall time may indicate that in these cells there may be important influences on action potential fall time other than $Na_v1.9$. No correlations were seen for neurons with lower (–40 to –50 mV) membrane potentials (4 C-fiber units, 9 Aδ-units, and 6 Aα/β units).

DISCUSSION

This study has provided the first direct evidence that the $Na_v1.9$ Na^+ channel is expressed selectively in nociceptive-type neurons

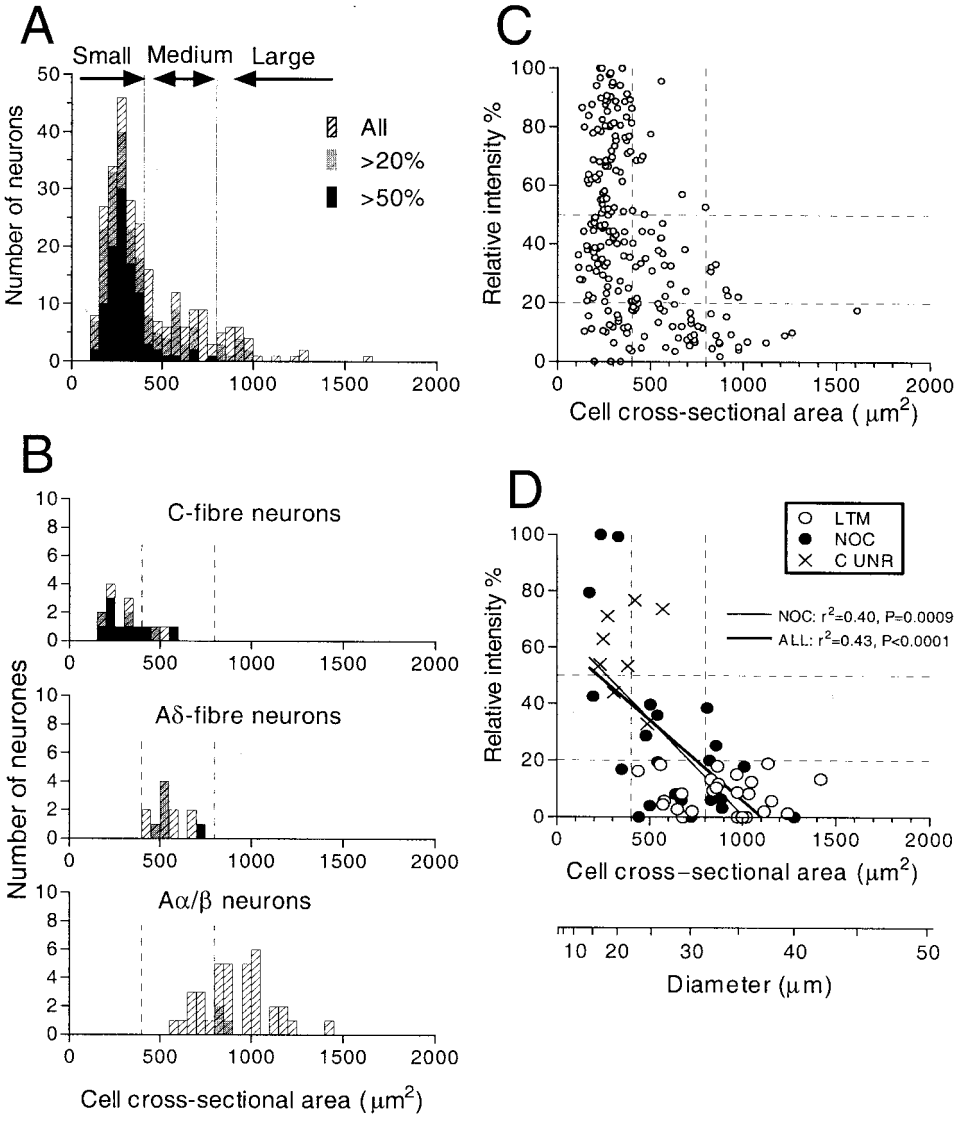

Figure 4. Relationship of $Na_v1.9$-LI relative intensity to neuronal cross-sectional area. In *A*, this is shown for all neurons (see Materials and Methods) from an L4 DRG from one of the experimental animals; *open bars* show all neuronal profiles with nuclei, *gray bars* show those with relative intensity >20%, and *black bars* show those with relative intensity >50%. In *B*, separate cell size distributions for dye-injected neurons with C-, Aδ-, or Aα/β-fiber neurons are shown in *B*. The *symbols* in *A* apply to both *A* and *B*. *C* shows an *x–y* plot of cell size against $Na_v1.9$-LI relative intensity for the same neurons as in *A*. In *D*, a similar *x–y* plot is shown for the dye-injected neurons with nociceptive (*NOC*), unresponsive (*UNR*), and for the low-threshold mechanoreceptive (*LTM*) properties. Linear regression analysis was performed for all neurons, for LTM, and for nociceptive neurons. The regression line, p, and r^2 values are given where a significant ($p < 0.05$) linear correlation was found.

(A-fiber nociceptive and C-fiber nociceptive and unresponsive units), but not in LTM neurons. As expected, $Na_v1.9$-LI intensity was correlated negatively with soma size in nociceptive neurons. In addition, it was correlated negatively with dorsal root conduction velocity in all nociceptive neurons and, especially clearly, in C-fiber neurons. It was correlated positively with somatic action potential duration in nociceptive-type neurons, with a stronger overall correlation with action potential rise time than fall time. However, no correlations with action potential duration were present in units with low membrane potentials (–40 to –50 mV).

The greater intensity of $Na_v1.9$ immunostaining in small neurons is consistent with previous studies of $Na_v1.9$ immunoreactivity. However, positive staining in some medium-to-large cells reported here, although consistent with a previous study of $Na_v1.9$ mRNA (Dib-Hajj et al., 1998), was not reported in several other studies of the mRNA and protein in these neurons (Tate et al., 1998; Amaya et al., 2000). This apparent discrepancy probably results from differences in positive/negative borderlines in the different studies. This is illustrated by our comparison of the effect of using positive/negative borderlines of 50 and 20% relative intensity. When we use 50%, only strongly labeled cells are classified as positive, and these are all within the small size range, whereas when we use 20%, some less intensely labeled cells also are classified as positive, including cells in the medium and large size range. It is likely that some of these less intensely positive cells (between 20 and 50% relative intensity) may not have been counted as positive in some previous studies.

$Na_v1.9$-LI in relation to functional membrane channels:

In a strict sense we cannot assume that the functional membrane properties in the soma or fiber should be linked directly to the presence of a channel protein in the cytoplasm of the cell, because the former depend on insertion of the protein into the membrane and on all conditions for activation of the channel being fulfilled. Our results provide a measure of cytoplasmic $Na_v1.9$-LI (possibly together with membrane-associated $Na_v1.9$-LI), but not specifically of membrane-inserted $Na_v1.9$-LI. Nonetheless, the correlations between cytoplasmic $Na_v1.9$-LI and certain somatic and fiber membrane properties (see below) do support such a link in the case of $Na_v1.9$ in DRG neurons. Furthermore, our findings are consistent with previous findings of $Na_v1.9$-LI along many IB4-positive C-fibers and at some nodes of Ranvier of thinly myelinated Aδ-fibers of the sciatic nerve, but rarely in large-diameter Aα/β-fibers (Fjell et al., 2000; Liu et al.,

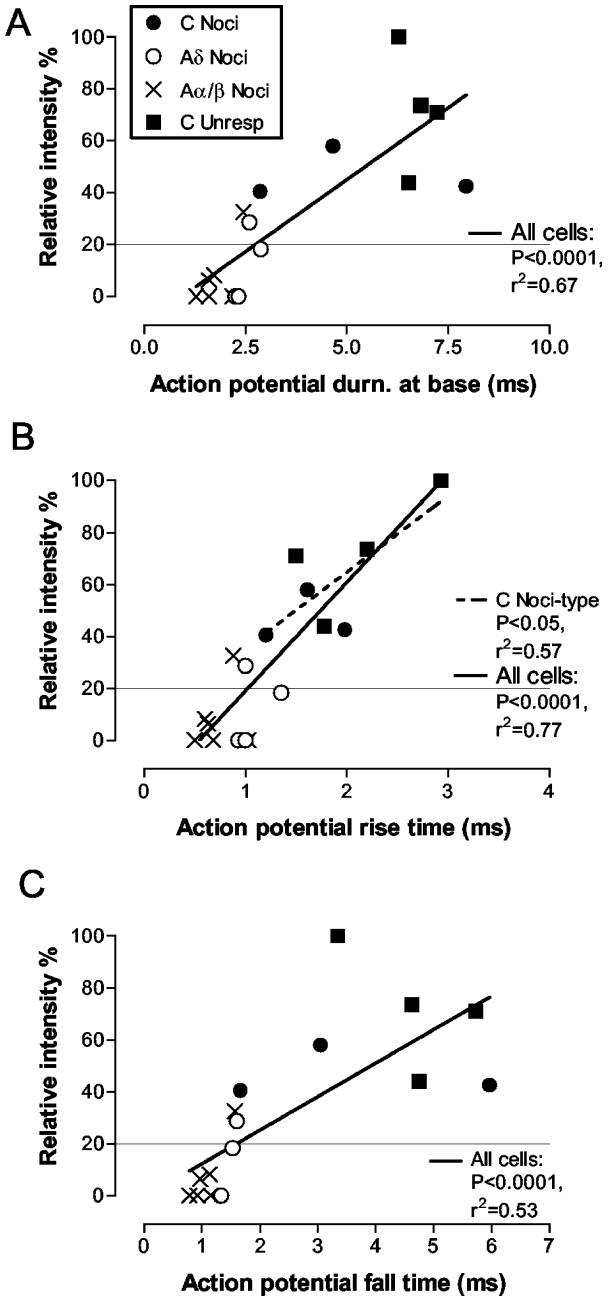

Figure 5. Relationship of $Na_v1.9$-LI relative intensity to membrane properties in nociceptive-type neurons for units with membrane potentials equal to or more negative than –50 mV. The *symbols* for all graphs are shown in *A*. *A*, $Na_v1.9$-LI versus action potential duration. *B*, $Na_v1.9$-LI versus action potential rise time. *C*, $Na_v1.9$-LI versus action potential fall time. *C Noci-type* means C-nociceptive plus C-unresponsive units. The regression line, *p*, and r^2 values are given where a significant ($p < 0.05$) linear correlation was found.

2001). This shows that $Na_v1.9$ is present in fibers of all of the expected diameters and supports the possibility that the cytoplasmic $Na_v1.9$-LI may be related to the availability of $Na_v1.9$ subunits for insertion into the membrane.

Nav1.9-LI in physiologically identified DRG neurons

Nav1.9 had been thought to be localized in nociceptive neurons on the basis of its presence in small-sized DRG neurons (Dib-Hajj et al., 1998) and its colocalization with markers generally assumed to be specific for nociceptors (VR1, trkA, and IB4) (Amaya et al., 2000). However, without direct examination of the sensory receptive properties of neurons to see whether they displayed $Na_v1.9$-LI, it was not known whether only nociceptive neurons expressed $Na_v1.9$, and, if expressed by nociceptive neurons, whether both C- and A-fiber nociceptive neurons expressed it. Our studies have confirmed the presence of $Na_v1.9$ not only in C-nociceptive-type (nociceptive and unresponsive) neurons but also in Aδ- and Aα/β-fiber nociceptive neurons; moreover, these studies also have confirmed the absence of $Na_v1.9$ in LTM neurons.

Unresponsive afferent units, sometimes called silent nociceptors, have been found projecting to joint or skin (Bessou and Perl, 1969; Handwerker et al., 1991; Djouhri et al., 1998). At least some of these respond, either after repeated stimuli (Handwerker et al., 1991) or during inflammation of the tissues (Meyer et al., 1991; Schmidt, 1996), to stimuli that previously did not excite them. It therefore has been suggested that they become sensitized by tissue damage or inflammation, thus lowering their thresholds into the range of normal nociceptive neurons (Schmidt, 1996). In the present study the C-unresponsive units displayed action potential characteristics very similar to those of C-nociceptive units and thus are considered to be probable high-threshold silent nociceptive units and/or nociceptive units with inaccessible receptive fields. The presence of $Na_v1.9$ in C-unresponsive neurons thus is consistent with its presence only in high-threshold/nociceptive neurons.

Conduction velocity

We found that, compared with $Na_v1.9$-LI-negative neurons, $Na_v1.9$-LI-positive neurons had slower dorsal root conduction velocities, especially in C-fiber neurons. Although the mechanisms by which $Na_v1.9$ may contribute to the conduction of impulses along the fiber are unknown, it is possible that persistent $Na_v1.9$ currents at resting potential could cause depolarization of the fiber. Although it might be argued that in $Na_v1.9$-positive cells there are differences in the levels of expression of other channels (e.g., TTX-S channels) or pumps that affect conduction velocity, computer simulations suggest that the inclusion of $Na_v1.9$ in the cell membrane can have a substantial (10–20 mV) depolarizing effect (Herzog et al., 2001), which may cause inactivation of low-threshold TTX-S Na^+ channels such as $Na_v1.7$ (PN1). Fewer available low-threshold Na^+ channels would reduce the rate of depolarization, and this would slow the fiber conduction velocity.

Action potential duration

DRG neurons with more intense $Na_v1.9$-LI tended to have longer action potential durations. Broad action potentials are characteristic of nociceptive DRG neurons *in vivo* (Koerber and Mendell, 1992; Ritter and Mendell, 1992; Djouhri et al., 1998; Gee et al., 1999), and in the present study we have found $Na_v1.9$-LI also to be characteristic of nociceptive-type neurons. It therefore seems likely that $Na_v1.9$ in nociceptive neurons may contribute to the prolonged action potential duration in these neurons. This relationship is stronger for action potential rise time than fall time as would be expected for an effect that, by depolarizing the membrane, caused the inactivation of Na^+ channels with fast kinetics, thus slowing the action potential depolarization. Herzog et al. (2001) showed that the increase in duration of the somatic action potential is mainly a result of the depolarizing influence of $Na_v1.9$ on resting membrane potential. The lack of correlation in units with low membrane potentials (–40 to –50 mV) may prove to be

related to the ultraslow inactivation of $Na_v1.9$ (Cummins et al., 1999). Although the correlation coefficients between $Na_v1.9$ and action potential rise time are relatively high, there was no such correlation of $Na_v1.9$ intensity in C-fiber neurons with action potential fall time. The slow kinetics of the TTX-R channel $Na_v1.8$, thought to be carrying much of the inward current in DRG neurons with broad action potentials, also must contribute to the slow kinetics of the prolonged action potentials (Renganathan et al., 2000). Indeed, it may be the interaction of the effects of these two channels that is important. Irrespective of this, the pattern of expression of $Na_v1.9$ in A-fiber nociceptive and C-nociceptive-type DRG neurons seen in the present study is consistent with the suggestion that $Na_v1.9$ may contribute to the longer action potential duration in nociceptive neurons and to the slower conduction velocities, especially in C-fiber neurons (Fjell et al., 2000).

Conclusion

In summary, $Na_v1.9$-LI was most intense in the smallest, most slowly conducting sensory neurons. It was expressed in nociceptive-type C-fiber neurons and in nociceptive Aδ- and Aα/β-fiber units, consistent with previous findings of $Na_v1.9$ in C-fibers, some Aδ-fibers, and a few Aα/β-fibers (Fjell et al., 2000). It was not, however, found in LTM units. The selective expression of $Na_v1.9$ within nociceptive, but not LTM, neurons means that any function of $Na_v1.9$ is restricted to nociceptive neurons. $Na_v1.9$-LI intensity in C-fiber neurons is correlated clearly with fiber conduction velocity, suggesting that $Na_v1.9$ may decrease C-fiber conduction velocity. In addition, in nociceptive-type neurons $Na_v1.9$-LI was correlated with action potential duration, especially action potential rise time. Although confirmation of the role of $Na_v1.9$ in influencing conduction velocity or action potential duration will require studies that use the selective antagonists of $Na_v1.9$, which are not yet available, or gene targeting technology to alter $Na_v1.9$ expression, the present findings demonstrate the selective expression of $Na_v1.9$ in nociceptive neurons and support previous suggestions that $Na_v1.9$ may contribute to the membrane properties of nociceptive neurons and, therefore, to nociceptive transmission.

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