

Voltage-dependent sodium (Na_V) channels in group IV sensory afferents

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Abstract

Patients with intermittent claudication suffer from both muscle pain and an exacerbated exercise pressor reflex. Excitability of the group III and group IV afferent fibers mediating these functions is controlled in part by voltage-dependent sodium (Na_V) channels. We previously found tetrodotoxin-resistant $\text{Na}_V1.8$ channels to be the primary type in muscle afferent somata. However, action potentials in group III and IV afferent axons are blocked by TTX, supporting a minimal role of $\text{Na}_V1.8$ channels. To address these apparent differences in Na_V channel expression between axon and soma, we used immunohistochemistry to identify the Na_V channels expressed in group IV axons within the gastrocnemius muscle and the dorsal root ganglia sections. Positive labeling by an antibody against the neurofilament protein peripherin was used to identify group IV neurons and axons. We show that >67% of group IV fibers express $\text{Na}_V1.8$, $\text{Na}_V1.6$, or $\text{Na}_V1.7$. Interestingly, expression of $\text{Na}_V1.8$ channels in group IV somata was significantly higher than in the fibers, whereas there were no significant differences for either $\text{Na}_V1.6$ or $\text{Na}_V1.7$. When combined with previous work, our results suggest that $\text{Na}_V1.8$ channels are expressed in most group IV axons, but that, under normal conditions, $\text{Na}_V1.6$ and/or $\text{Na}_V1.7$ play a more important role in action potential generation to signal muscle pain and the exercise pressor reflex.

Keywords

Voltage-dependent sodium channels, dorsal root ganglia, exercise pressor reflex, muscle afferents

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Patients with peripheral artery disease often experience intermediate claudication (i.e. muscle pain), which results from atherosclerotic plaques in the major arteries of lower extremities.¹ It has been estimated that 12–18 million adults have peripheral artery disease and one-third of them experience claudication, which negatively impacts their quality of life.^{2,3} Along with pain, patients suffering from peripheral vascular disease can also experience an exaggerated exercise pressor reflex (EPR) that significantly increases their risk for myocardial infarction.⁴ In healthy individuals, the EPR is one of the mechanisms mediating cardiovascular adjustments to exercise.^{3,5}

Muscle nociceptive and EPR signals are transmitted along thinly myelinated group III and unmyelinated group IV axons.⁵ In patients with peripheral artery disease, poor muscle perfusion leads to ischemia, which is an activator of group III and IV afferents leading to muscle pain and an enhanced EPR.^{5,6}

The electrogenesis of these signals is mediated via voltage-gated sodium channels (Na_V). Dorsal root ganglia

(DRG) neurons have been reported to express several Na_V channel isoforms, including $\text{Na}_V1.1$, $\text{Na}_V1.6$ – $\text{Na}_V1.9$ channels.^{7,8} One method to distinguish Na_V channels is by their sensitivity to tetrodotoxin (TTX), with some channels such as $\text{Na}_V1.6$ and $\text{Na}_V1.7$ being TTX-sensitive (TTX-S), and others such as $\text{Na}_V1.8$ being resistant to the toxin (TTX-R). Using immunocytochemistry, we previously found evidence for $\text{Na}_V1.6$ – $\text{Na}_V1.9$ channel expression in isolated muscle afferent neurons, but not $\text{Na}_V1.1$.⁹ However, using TTX and other Na_V channel blockers, our electrophysiological results

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supported $\text{Na}_V1.8$ as the dominant Na_V channel in muscle afferent somata.^{9,10}

While TTX minimally affected somatic Na_V currents (average 14% block),⁹ axonal action potentials from both group III and IV afferents were almost completely blocked by TTX applied to the dorsal roots,¹¹ which supports little, if any, role in action potential generation by TTX-R channels, such as $\text{Na}_V1.8$. So, we were interested in knowing if $\text{Na}_V1.8$ channels are expressed in group IV fibers. In addition, since TTX cannot distinguish different TTX-S Na_V channels, we wanted to determine which TTX-S channels are expressed by these afferent neurons. To better understand the Na_V channel distribution patterns, we compared the expression of these channels by immunohistochemical staining of the muscle and DRG sections. Group IV neurons and axons were identified by labeling with a peripherin antibody.^{12,13} We show that $\text{Na}_V1.6$, $\text{Na}_V1.7$, and $\text{Na}_V1.8$ channels are expressed in >67% of muscle afferent axons and somata. While there were no significant differences in the percentage of axons vs. somata labeled by the $\text{Na}_V1.6$ and $\text{Na}_V1.7$ antibodies, the antibody targeting $\text{Na}_V1.8$ channels labeled significantly fewer group IV axons than somata, which fit well with the electrophysiological data. It seems possible that $\text{Na}_V1.8$ could play a role in action potential generation within group IV axons. However, the conditions under which that could occur have yet to be identified.

Experimental procedures

All animal protocols were approved by the Kirksville College of Osteopathic Medicine IACUC and were consistent with the National Research Council guide for the Care and use of Laboratory Animals. Adult male Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, PA) weighing between 150 and 400 g were used in these experiments. Rats were housed in an USDA-Approved, AAALAC-Certified Animal Care facility at a constant temperature of $24 \pm 1^\circ\text{C}$, controlled 12-h light dark cycles and access to commercial rat chow and water ad libitum.

Muscle preparation

Rats were sacrificed using CO_2 followed by decapitation using a laboratory guillotine (Kent Scientific Corp, Torrington, CT).⁹ Both gastrocnemius muscles were dissected out along with the tendons, washed in ice-cold PBS solution, and flash frozen in dry ice-cooled isopentane. The muscles were kept frozen at -80°C until use. Frozen muscles were cut longitudinally in 25 μm sections using a Leica CM1900 cryostat (Leica Microsystems Inc., Buffalo Grove, IL). The sections were mounted on polylysine-coated slides, allowed to dry and stored at -20°C until use.

DRG preparation

Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine, xylazine, and acepromazine, and both right and left gastrocnemius muscles were injected with 100 μl of 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Life Technologies, Grand Island, NY) solution (1.5% in DMSO) as previously described.⁹ Four to five days were allowed for transport of DiI from the muscle to the somata of muscle afferents in the DRG. Rats were sacrificed and the Lumbar 4 and 5 DRG were dissected out, frozen, and sectioned as described above.

Superior cervical ganglia preparation

Superior cervical ganglia (SCG) were dissected from sacrificed rats (as described for muscle preparation), frozen, and sectioned as described above to be used as control tissue to test the antibodies.

Staining

The slides were brought to room temperature and rehydrated with $1 \times$ PBS solution for 3 min. The sections were post-fixed with 4% formaldehyde and permeabilized with 0.2% triton X-100 (muscle sections) or 0.2% tween-20 (DRG and SCG sections). The slides were then incubated with blocking solution for 1 h, followed by overnight incubation with primary antibodies, which were chicken polyclonal anti-peripherin and a Na_V channel antibody, which was either rabbit polyclonal anti- $\text{Na}_V1.1$, rabbit polyclonal anti- $\text{Na}_V1.6$, mouse monoclonal anti- $\text{Na}_V1.7$, or mouse monoclonal anti- $\text{Na}_V1.8$ (Table 1). Following overnight incubation, the sections were washed with PBS and incubated for 1 h in the appropriate secondary antibodies, anti-chicken FITC (1:200, Aves Labs, Inc.), anti-rabbit Alexa Fluor 633 (1:250, Life Technologies), and/or anti-mouse Alexa Fluor 546 (1:500, Life Technologies). The sections were visualized and images captured using the Nikon epifluorescence microscope. The intensity of green fluorescent labeling appeared to be uniform throughout the fibers within an image, but both the red and blue fluorescence appeared to be less even within a single focal plane. However, adjusting that focal plane revealed that the fibers were just as evenly labeled by red and blue fluorescent as with the green fluorescence, which suggests that the depth of field was greater for imaging green fluorescence than for either red or blue fluorescence.

Quantification

Nerve fibers that were at least 100 μm long and positive for peripherin were counted and checked for colabeling by Na_V antibodies. The data are expressed as the percentage

Table 1. List of antibodies used for labeling.

Primary Ab	Type	Company	Conc. used
Peripherin	Anti-chicken	Aves Labs Inc	1:1000
Na _v 1.1	Anti-rabbit	Alomone labs	1:200
Na _v 1.6	Anti-rabbit	Abcam	1:200
Na _v 1.7	Anti-mouse	Neuromab	1:500
Na _v 1.8	Anti-mouse	Abcam	1:200

of peripherin labeled fibers that were positively stained by the Na_v antibody. Peripherin negative fibers were not counted. The data were compiled from gastrocnemius muscles from 3–4 rats for each Na_v antibody.

For DRG and SCG neuronal counts, images were captured at 10 \times , fluorescence intensity and cell size were measured from individual neurons using ImageJ (rsbweb.nih.gov/ij/index.html). The fluorescent intensity of unlabeled neurons was measured and plotted in a histogram to determine the threshold for positively labeled neurons as previously described.⁹ The DRG somata that were positively labeled with DiI and the peripherin antibody were considered as group IV muscle afferent neurons.

Results

To better understand the Na_v channels expressed in muscle afferent axons, we used immunohistochemistry to identify the expressed channels. We visualized group IV afferents by staining muscle sections with an antibody against peripherin, which is a neurofilament protein that is selectively expressed in unmyelinated (e.g. group IV) sensory axons.¹² For comparison with our electrophysiological data, we also examined Na_v channel expression in DRG sections. Muscle afferent neurons within these sections were identified by retrograde labeling using DiI that was injected into the gastrocnemius muscle, and group IV neurons were further identified by positive peripherin antibody labeling.

We previously defined group IV neurons as those with cell body diameters <30 μ m.^{9,10} This definition was tested by calculating the diameters of DiI and peripherin labeled (i.e. group IV) somata in the DRG sections. The diameters ranged from 16 to 35 μ m with an average of 23 \pm 4 μ m with 96% of group IV neurons having a diameter <30 μ m. (Figure 1). These results support our inclusion of neurons with diameters <30 μ m in group IV.

Na_v1.8 channels in group IV afferent fibers

Previous electrophysiology experiments supported a dominant role for Na_v1.8 channel activity in group III and IV somata,⁹ but a minimal role in the axons.¹¹ This

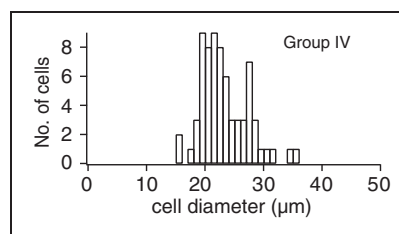


Figure 1. Neuronal diameter histogram of group IV neurons. Neurons from DRG sections that were labeled by both DiI and the peripherin antibody were counted as group IV.

suggested that Na_v1.8 channels may not be expressed in group IV axons. However, we found that group IV axons within gastrocnemius muscles were positively labeled by the Na_v1.8 channel antibody (Figure 2(a)). Labeling was found in individual group IV nerve fibers (Figure 2(a)) and in group IV fibers within nerve tracts (Figure 2(b)). No labeling was observed in control slides (Figure 2(c)), which was true for all experiments regardless of the antibody used (not shown). Individual peripherin-positive fibers were counted to determine the percentage of those fibers labeled by the Na_v1.8 antibody. On average, we found that 67 \pm 1% (mean \pm SD from three muscles) of the peripherin-positive fibers were labeled by the Na_v1.8 channel antibody (Table 2). The percentage of Na_v1.8-positive group IV fibers ranged from 66% to 68% for the three muscles tested with a total of 156 fibers counted (34–63 fibers/muscle). Each muscle came from a different animal. Interestingly, the counts from DRG sections showed that 96 \pm 7% (20/21) of group IV somata were labeled by the Na_v1.8 channel antibody, which was significantly higher than for group IV fibers (Table 2). Three DRG sections from each of three different animals were counted (total=9 sections). These results support the electrophysiological results that showing Na_v1.8 channels play a larger role in somatic vs. axonal Na⁺ influx.^{9,11}

TTX-S Na_v channels in group IV axons and somata

Previous electrophysiology studies demonstrated that muscle afferent somata⁹ and axons¹¹ express TTX-S Na_v channels. In addition, we showed that antibodies directed against Na_v1.6 and Na_v1.7 channels could label isolated muscle afferent neurons.⁹ However, the expression of these TTX-S Na_v channels in muscle afferent axons has not been investigated. We colabeled muscle afferents with peripherin and antibodies targeting Na_v1.1, Na_v1.6, or Na_v1.7 channels.⁷ Similar to our previous study of muscle afferent somata,⁹ we failed to find any Na_v1.1-positive fibers (Figure 3). Since we did not find Na_v1.1 channel labeling in either cultured cells or muscle afferent axons, we did not further investigate this channel in DRG sections.

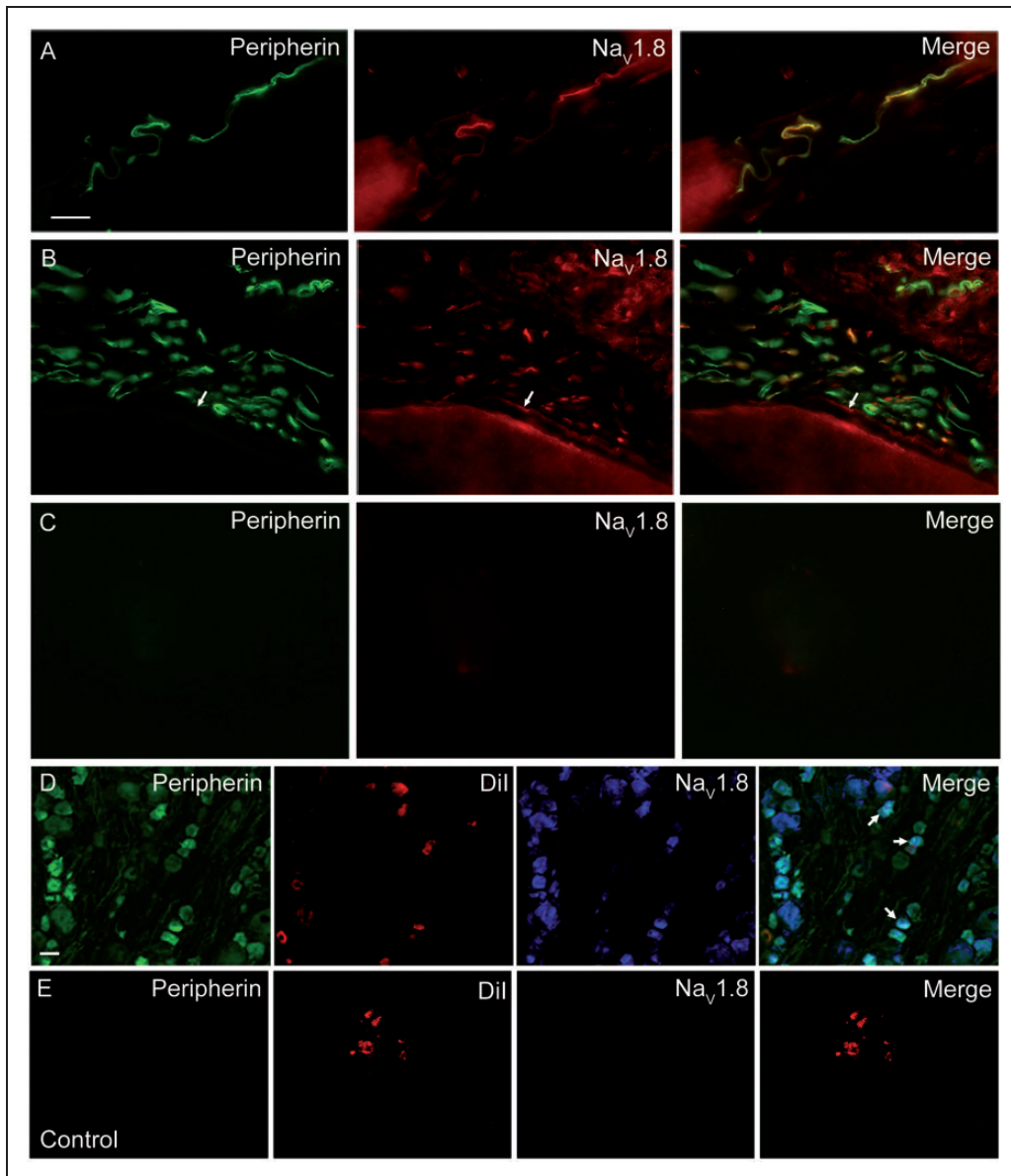


Figure 2. $\text{Na}_V1.8$ antibody labeling in muscle and DRG sections. Muscle and DRG sections were colabeled with a mouse $\text{Na}_V1.8$ channel antibody and a chicken peripherin antibody. $\text{Na}_V1.8$ channels are present in peripherin-positive group IV nerve fibers (a) and also in the nerve bundles (b). $\text{Na}_V1.8$ channel antibody also labeled some of the peripherin-negative fibers (arrow). (c) Muscle sections exposed only to the secondary antibodies (control) showed no labeling, which was consistently found in all experiments. (d) DRG sections showing that group IV neurons (colabeled with Dil and the peripherin antibody) are also labeled by the $\text{Na}_V1.8$ antibody (arrows). (e) DRG sections exposed only to the secondary antibodies (negative control) showed only Dil labeled neurons. Scale = 50 μm .

$\text{Na}_V1.6$ channels are widely expressed in sensory neurons, including unmyelinated sensory fibers.^{14,15} The $\text{Na}_V1.6$ channel antibody positively labeled $84 \pm 9\%$ of peripherin-positive fibers (Table 2; Figure 4(a) and (b)). For the gastrocnemius muscles from the three animals tested, the percentage of $\text{Na}_V1.6$ -positive group IV axons ranged from 74% to 93% from a total of 208 group IV fibers counted. As with $\text{Na}_V1.8$, $\text{Na}_V1.6$ channel labeling was found in both individual group IV fibers and those within nerves (Figure 4(a) and (b)). We consistently saw

a light, diffuse muscle fiber staining by the $\text{Na}_V1.6$ channel antibody, which may result from non-specific myosin labeling.¹⁶ In the DRG sections, $98 \pm 4\%$ (21/22) of the muscle afferent somata were labeled by the $\text{Na}_V1.6$ channel antibody (Figure 4(c)). There was no statistical difference between the percentages of $\text{Na}_V1.6$ -positive group IV axons vs. somata (Table 2).

$\text{Na}_V1.7$ channel activity is important for action potential generation and propagation in nociceptors.^{17,18} Within the peripherin-positive group IV fibers, we found

Table 2. Nav antibody labeling of group IV afferents.

Nav subtype	Fibers		Somata	
	No. of fibers	% positive	No. of neurons	% positive
Nav1.6	175/208	84 ± 9	21/22	98 ± 4
Nav1.7	140/185	77 ± 6	23/27	82 ± 21
Nav1.8	104/156	67 ± 1	20/21	96 ± 7 ^a

^aSignificant difference between somal and axonal labeling.

that 77 ± 6% were labeled by the Nav1.7 antibody (Table 2; Figure 5), which included both individual fibers (Figure 4(a)) and fibers within axon bundles (Figure 4(b)). For gastrocnemius muscles from the three animals tested, between 71% and 83% of group IV fibers were labeled by the Nav1.7 antibody with a total of 185 group IV axons counted. As with Nav1.6, we saw a diffuse labeling of muscle fibers with the Nav1.7 antibody, which may result from non-specific myosin labeling.¹⁶

In the DRG sections, 82 ± 20% (23/27) of the group IV somata were Nav1.7-positive, which was not significantly different from the percentage of Nav1.7-positive group IV fibers. As a result of the high percentage of axons

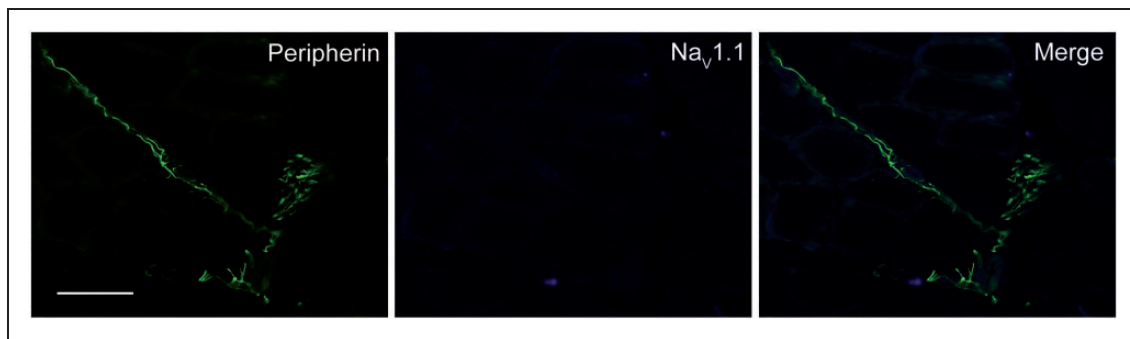


Figure 3. An antibody against Nav1.1 channels fails to label muscle afferent axons in gastrocnemius muscle. Peripherin-positive group IV axons are shown, but we never found muscle afferent fibers co-labeled by the Nav1.1 channel antibody. Scale = 50 μm.

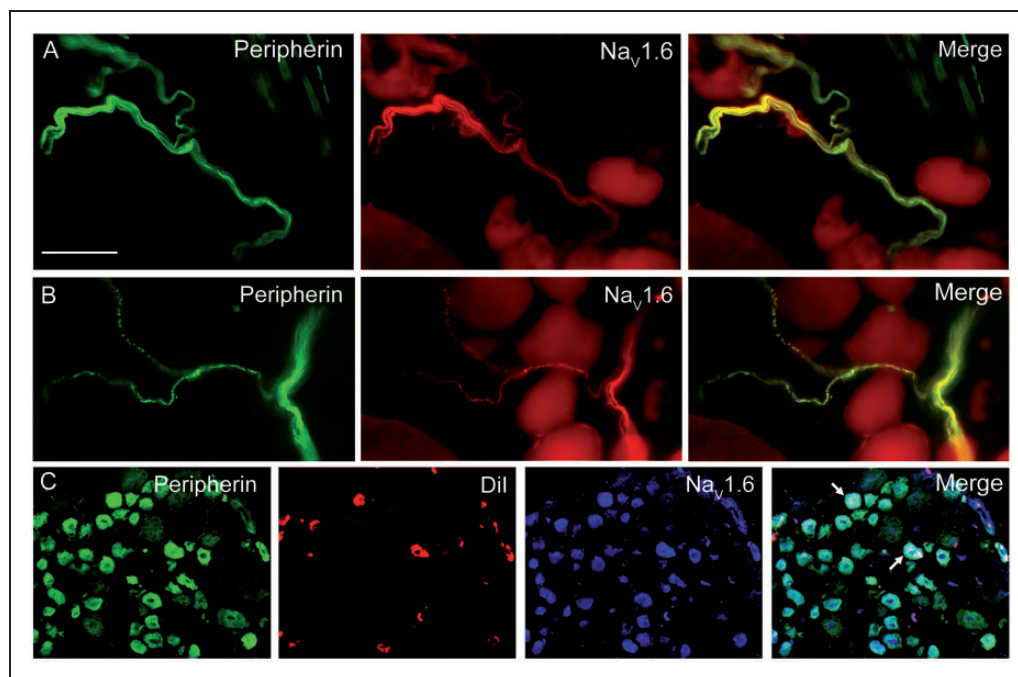


Figure 4. A Nav1.6 channel antibody labels group IV fibers in muscle sections and neurons in DRG sections. Gastrocnemius and DRG sections were colabeled with a rabbit Nav1.6 channel antibody and a chicken peripherin antibody. Nav1.6 channels were present in peripherin-positive group IV nerve fibers (a, b). Some of the peripherin-positive fibers are not stained positive for Nav1.6 (a, upper right corner). (c) Most DRG group IV somata are labeled by the Nav1.6 channel antibody (arrows). Scale = 50 μm.

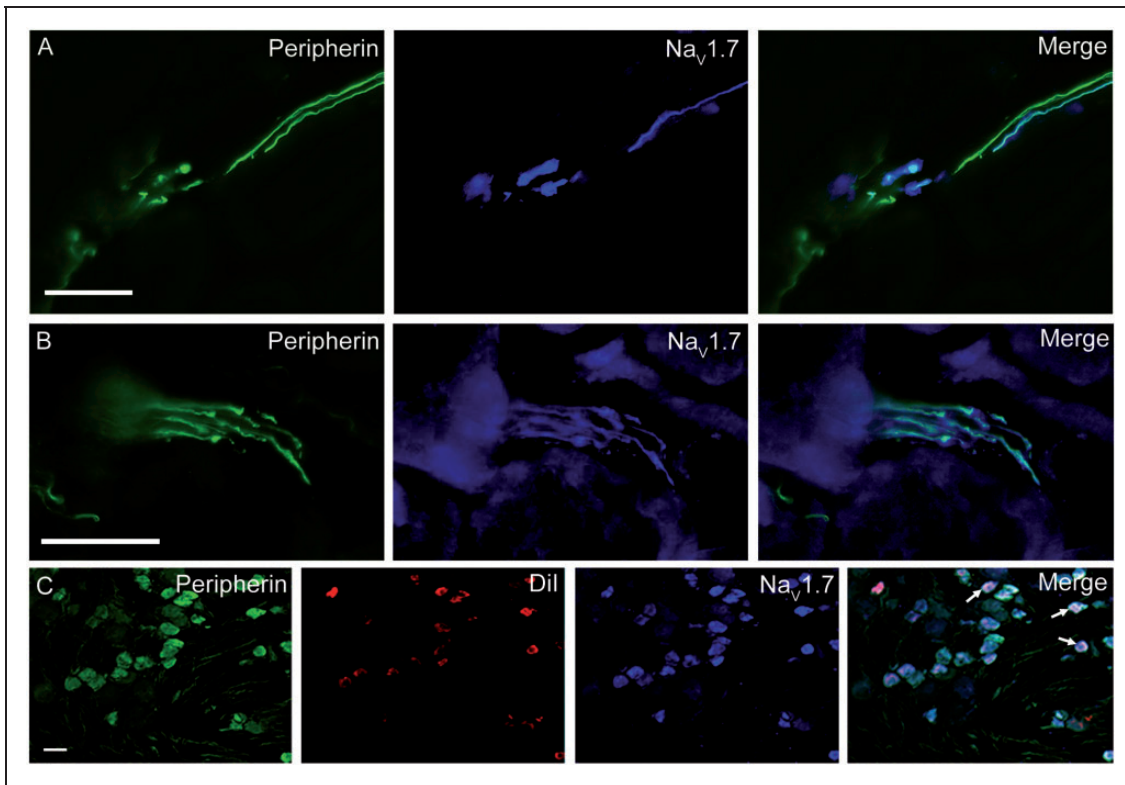


Figure 5. Group IV fibers and neurons are labeled by a Na_v1.7 channel antibody. Gastrocnemius and DRG sections were colabeled with a mouse Na_v1.7 channel antibody and chicken peripherin antibody. Na_v1.7 channels are expressed in by most individual group IV axons (a) as well as those in nerve bundles (b). However, some group IV axons failed to show evidence of Na_v1.7 channel labeling (panel a). (c) The majority of peripherin-positive, Dil-positive (group IV) neurons were also positive for Na_v1.7 channel antibody labeling (arrows). Scale = 50 μm.

expressing these TTX-S channels, it is likely that both Na_v1.6 and Na_v1.7 channels contribute to the TTX-S Na⁺ influx in both group IV axons and somata.

SCG labeling

As a test for Na_v antibody specificity, we examined antibody labeling of SCG neurons, which express Na_v1.6 and Na_v1.7, but not Na_v1.8 channels.^{19,20} Sympathetic neurons are B type, so the peripherin antibody did not label these cells (Figure 6 (a), (c), and (e)). For Na_v antibody labeling, the Na_v1.6 antibody labeled 91% (Figure 6(b) and (g)) and Na_v1.7 antibody labeled 95% of neurons in the SCG (>100 cells for each antibody tested) (Figure 6(d) and (h)). Histograms of fluorescent intensity show peaks that were statistically distinct from baseline labeling (Figure 6 (g) and (i)). However, the histogram for Na_v1.8 antibody labeling shows complete overlap with background, which demonstrates no labeling by this antibody (Figure 6(f) and (h)), as expected.²⁰

Discussion

The Na_v isoforms that generate action potentials in group IV afferents are not known, but previous reports

suggested that the dominant isoforms could differ between muscle afferent somata vs. axons.^{9,11} Using the peripherin antibody, we were able to label a subset of axons within rat gastrocnemius muscles, which based on previous publications we identified as unmyelinated group IV afferents.^{12–14,21} The majority of group IV afferent axons were positively labeled by antibodies to either Na_v1.8 (67%), Na_v1.6 (83%), or Na_v1.7 (77%) channels. A diffuse muscle staining was observed with both the Na_v1.6 and Na_v1.7 channel antibodies. While both Na_v1.6 and Na_v1.7 channels are reported to be expressed in non-neuronal tissues,^{22–25} these channels have not been found in skeletal muscle fibers.^{26,27} Na_v1.7 channel antibodies have been previously observed to label skeletal muscle proteins, but this was interpreted as non-specific labeling of myosin, since the labeling was not changed when the antibody was pre-blocked with peptide.¹⁶ Thus, the diffuse muscle labeling likely results from non-specific absorption of these antibodies by myosin.¹⁶

Within the DRG, a significantly larger percentage of group IV somata were labeled by the Na_v1.8 channel antibody relative to afferent fibers, but there was no statistical difference between axonal and somata labeling for antibodies to either Na_v1.6 or Na_v1.7 channels. Given

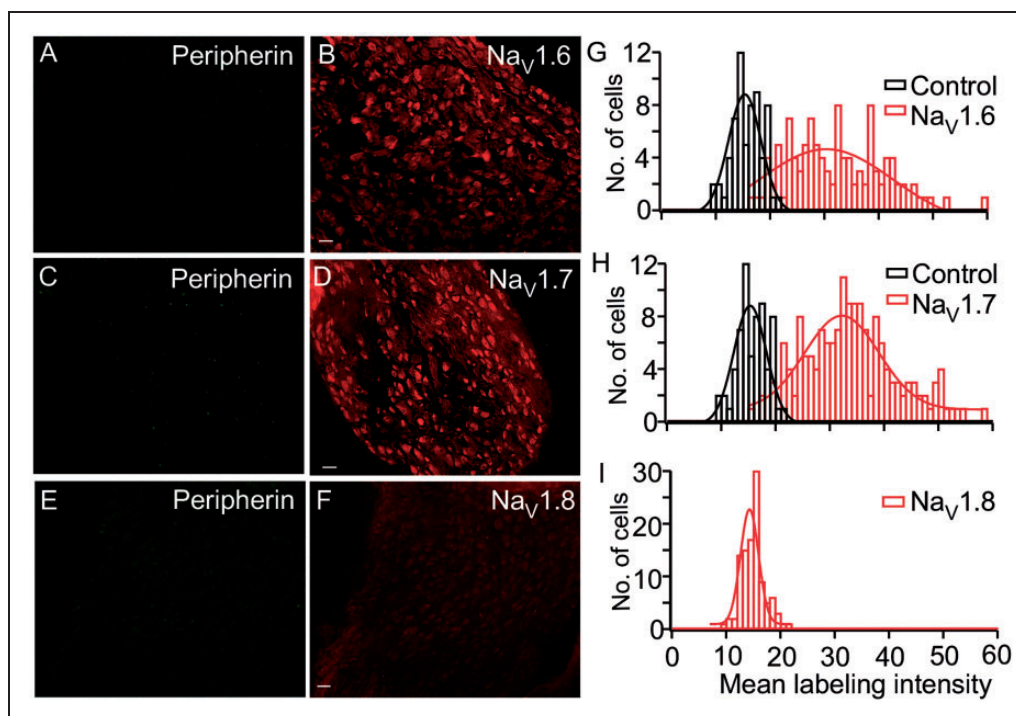


Figure 6. SCG labeled with Na_v antibodies. As expected, the peripherin antibody failed to label SCG neurons or fibers (a, c, e). Na_v1.6 channel antibody labeled most neurons. Note that some neurons were labeled more intensely than others (b). Almost all cells showed intense labeling by Na_v1.7 channel antibody (d). Na_v1.8 channel antibody failed to label any SCG neurons (f). Histograms of the fluorescent intensity show background (Bkgd, black bars) and the Na_v antibody labeling (red bars). The histograms were fit using a Gaussian equation (g-i). At least 100 SCG neurons were counted for each Na_v antibody tested.

the high percentage of Na_v-positive fibers and somata, it appears that a majority express at least two of these Na_v channel isoforms, and many may express all three. If these channels are functional in the axon, it is likely that all three participate in AP generation within group IV axons to signal muscle pain and the EPR.

Na_v1.1

We have been unable to produce evidence for Na_v1.1 channel expression in muscle afferents, including group IV muscle afferent fibers and muscle afferent somata.⁹ These findings are not surprising since Na_v1.1 channel antibodies failed to label cutaneous afferents and only weak labeling was observed in isolated somata.²¹ mRNA for Na_v1.1 channels can be detected in sensory afferents,^{28,29} which suggests that these channels could be produced. However, Na_v1.1 channel levels may be too low to be detected in our assay. Alternatively, Na_v1.1 channels have been reported to be only expressed in large diameter myelinated sensory neurons,³⁰ which were not studied here. A study using conotoxins that blocked a range of Na_v channels concluded that Na_v1.1 channels did not significantly contribute to action potential generation in either C-fiber (group IV) or Aδ-fibers (group III) in sciatic nerve.³¹

Na_v1.6

Several reports have demonstrated that TTX-S Na_v1.6 channels are present at the nodes of Ranvier in myelinated nerve fibers and play a role in saltatory conduction,³² but Na_v1.6 channel expression has also been identified in unmyelinated sensory neurons and may play an important role in pain signaling. Indeed, previous reports showed that 100% of peripherin-positive fibers within sciatic nerve were positive for Na_v1.6 channels.^{14,30} Similarly, we found Na_v1.6 expressed in 84% of the group IV (unmyelinated) nerve fibers and 96% of somata. However, the role in group IV action potential generation may be limited. The transient knockdown of Na_v1.6 channels was found to have only a small effect on C-fiber excitability, while strongly reducing Aδ fiber excitability.¹⁵ In addition, Na_v1.6-targeted conotoxins failed to affect compound action potentials in C-fibers (group IV) but completely blocked Aδ-fiber (Group III) action potentials in sciatic nerve.³¹ Thus, the loss of functional Na_v1.6 channels does not appear to change excitability of unmyelinated sensory afferents (C-fibers and group IV fibers). Interestingly, Na_v1.6 knockdown reduced pain behavior in the animals, which led to the conclusion that Na_v1.6 could be an important analgesic drug target.¹⁵ The role of Na_v1.6

channels in controlling group IV axonal excitability requires further investigation.

Na_v1.7

The role of Na_v1.7 channels in human nociception has been well established based on the effects of Na_v1.7 channel mutations on human pain perception.^{18,30,33–35} Therefore, it is not surprising that we found the majority of group IV neurons and fibers labeled by the Na_v1.7 channel antibody. However, a previous study showed a lower percentage of Na_v1.7-positive C-fibers in sciatic nerve (27%) vs. DRG neurons (63%),¹³ and these percentages are lower than we found for group IV fibers (77%) and neurons (82%). The source of these differences is not clear, but some possibilities include differences (1) in Na_v channel expression between cutaneous and muscle afferents, (2) in Na_v channel expression between terminal fibers and those within the sciatic nerve, and (3) technical differences perhaps involving Na_v antibody penetration of individual fibers vs. those within the sciatic nerve. The available evidence appears to support the third possibility since toxins that target Na_v1.7 channels strongly inhibit the C-fiber component of the compound action potential.^{31,36} The strong expression by group IV afferents suggests Na_v1.7 channels will play an important role in muscle pain and the EPR. Indeed, the Na_v1.7 channel blocker Ssm6a significantly suppressed the muscle contraction-induced pressor effect mediated by the EPR.³⁶

Na_v1.8

The TTX-R Na_v1.8 channels are dominantly expressed in a majority of muscle afferent somata,⁹ and these channels have been shown to play an important role in action potential generation in nociceptor somata.^{7,37,38} Thus, it is possible that Na_v1.8 channels play a role in muscle nociception.^{21,39} We found 67% of the group IV fibers were positively labeled with the Na_v1.8 antibody, which is a significantly smaller percentage than for group IV somata (96%). This is in line with electrophysiological studies showing that Na_v1.8 channels provide the dominant pathway for Na⁺ influx in muscle afferent neurons⁹ but may not have sufficient axonal activity to support action potential propagation in group IV afferents.¹¹ Indeed, muscle specific group III and IV signals are almost completely blocked by TTX,¹¹ which indicates that Na_v1.8 channels play a minimal role.

Although not a focus of this study, we found that non-peripherin labeled fibers could also be labeled by the Na_v1.8 antibody. This supports the presence of Na_v1.8 channels in non-group IV afferents and reinforces our earlier studies that supported expression of Na_v1.8 channels in small, medium, and large diameter afferent neurons.^{9,10}

Using immunohistochemistry, Na_v1.8 channels have been previously shown to be expressed by the majority of sensory free nerve endings in the skin.²¹ Indeed, 96% of these cutaneous endings were positive for Na_v1.8 channels, which is a higher percentage than we found in muscle. However, we used peripherin to selectively examine unmyelinated sensory fibers, while a PGP9.5 antibody were used to identify afferent axons in the skin,²¹ and PGP9.5 is a marker for both unmyelinated and myelinated sensory afferents.⁴⁰ Thus, one possible explanation for the higher percentage of labeled cutaneous sensory fibers is that Na_v1.8 channels are more highly expressed by myelinated axons. An additional possibility is that Na_v1.8 channels are indeed expressed by a higher percentage of cutaneous afferent fibers than muscle afferent fibers. One potential reason for such a difference is that Na_v1.8 channel activity helps to sustain action potential firing at lower temperatures,⁴¹ and the skin is likely to experience much larger temperature changes than muscle.

Na_v channels mediating muscle pain

Pathological conditions have been shown to alter the expression and axonal distribution of Na_v channels,⁴² which could be important for mediating muscle afferent hyperexcitability. Indeed, Na_v1.7 and Na_v1.8 channels are involved in inflammatory pain,⁴³ and it is known that mutations of these channels are associated with neuropathic pain in human patients.^{34,44} It is interesting that hyperexcitability resulting from a mutant Na_v1.7 channel has been found to require Na_v1.8 channel expression.²⁰ The Na_v1.7 channel mutant, L858H, produces pain in response to mild warmth in humans and induces hyperactivity when expressed in DRG, but not in SCG.²⁰ However, coexpressing Na_v1.8 in SCG along with L858H produces SCG hyperactivity. It is thought that the more depolarized voltage dependence of activation and inactivation of Na_v1.8 channels³⁸ is a major determinant factor in producing nociceptor hyperactivity and thermal allodynia in patient carrying L858H mutation.²⁰

The Na_v1.8 channel expression has been shown to be increased in PGP9.5 labeled muscle nerve fibers in patients with chronic myofascial temporomandibular disorders.⁴⁵ However, a claudication animal model (peripheral vascular disease-reduced muscle blood flow) produced no changes in TTX sensitivity of group III/IV afferent activity,¹¹ which suggests that TTX-R channel activity (i.e. Na_v1.8) is not upregulated under muscle ischemia conditions. Potential changes in TTX-S channel activity (e.g. Na_v1.7) have not yet been investigated using this animal model. One caveat is that the ischemia duration produced by this animal model is three days, which may be too short to observe changes in axonal Na_v channel expression. Previous work showing changes in Na_v channel expression were done seven days

following nerve injury.^{42,46} Thus, it is possible changes in group III/IV Na_v channel expression could be observed in a longer duration muscle ischemia model.

Group IV fibers carry the action potentials that signal the EPR and muscle pain,⁵ and the evidence supports the involvement of multiple Na_v channels in action potential generation in pain fibers,²¹ as well as other sensory afferent fibers.^{47–49} A large fraction of group IV fibers express the Na_v channels, with 67% expressing Na_v1.8 channels, 84% expressing Na_v1.6 channels, and 77% expressing Na_v1.7 channels. Thus, it is highly likely that the majority of group IV fibers expressed at least two Na_v channel isoforms, and the expression of all three channel isoforms within single fibers could be common. Although we have not determined which of these channels participate in action potential generation, recent studies from the Kaufman group supports the involvement of multiple TTX-S channels, with a minimal role for TTX-R Na_v1.8 channels. Muscle afferent signals that generate the contraction-induced pressor response are almost completely blocked by TTX, while the Na_v1.8-specific blocker A803467 had little or no significant effect.¹¹ The Na_v1.7 channel blocker, Ssm6a, blocked these muscle specific signals by ~50%,³⁶ which, combined with the TTX results, suggests a substantial role for another TTX-S channel type, such as Na_v1.6. One issue is that the contraction-induced pressor signals are carried by both group III and IV neurons, so it is possible that the Ssm6a resistant signals are mediated by group III axons. However, Ssm6a partially blocked the compound action potentials recorded from both group III-A δ and group IV-C fibers,³⁶ which supports Na_v1.7 channel expression in both small myelinated (group III-A δ) and unmyelinated axons (group IV-C). Thus, it appears that both Na_v1.6 and Na_v1.7 channel participate in action potential generation in both group III and IV afferents, but Na_v1.8 channels, while present, may not have sufficient density to sustain action potential generation under normal conditions.

Declaration of Conflicting Interests

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