Comprehensive phenotyping of group III and IV muscle afferents in mouse

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Jankowski MP, Rau KK, Ekmann KM, Anderson CE, Koerber HR. Comprehensive phenotyping of group III and IV muscle afferents in mouse. J Neurophysiol 109: 2374–2381, 2013. First published February 20, 2013; doi:10.1152/jn.01067.2012.—While much is known about the functional properties of cutaneous nociceptors, relatively little is known about the comprehensive functional properties of group III and IV muscle afferents. We have developed a mouse ex vivo forepaw muscle, median and ulnar nerve, dorsal root ganglion (DRG), spinal cord recording preparation to examine the functional response properties, neurochemical phenotypes, and spinal projections of individual muscle afferents. We found that the majority of group III and IV muscle afferents were chemosensitive (52%) while only 34% responded to mechanical stimulation and fewer (32%) responded to thermal stimuli. The chemosensitive afferents could be grouped into those that responded to a “low”-metabolite mixture containing amounts of lactate and ATP at pH 7.0 simulating levels observed in muscle during exercise (metaboreceptors) and a “high”-metabolite mixture containing lactic acid concentrations and ATP at pH 6.6 mimicking levels observed during ischemic contractions (metabo-nociceptors). While the majority of the metabo-nociceptive fibers responding to the higher concentration levels were found to contain acid-sensing ion channel 3 (ASIC3) and/or transient receptor potential vanilloid type 1 (TRPV1), metaboreceptors responding to the lower concentration levels lacked these receptors. Anatomically, group III muscle afferents were found to have projections into laminae I and IIo, and deeper laminae in the spinal cord, while all functional types of group IV muscle afferents projected primarily into both laminae I and II. These results provide novel information about the variety of sensory afferents innervating the muscle and provide insight into the types of fibers that may exhibit plasticity after injuries.

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One of the most common complaints of patients suffering from chronic pain is musculoskeletal in nature. In fact, relatively few patients seek medical treatment for pain that is cutaneous in origin (Mense 2003). Several studies have focused on the role of muscle sensory neurons in various pain states and have shown that muscle injury (Dina et al. 2010; Sluka et al. 2003; Xu et al. 2010) and inflammation (Gautam et al. 2010; Sluka and Rasmussen 2010; Walder et al. 2010) result in sensitization of muscle nociceptors, which are correlated with increased pain behaviors (Alvarez et al. 2010; Chen et al. 2010; Dina et al. 2008; Ikewuchi et al. 2008).

While these studies and others (e.g., Ellrich and Makowska 2007; Hoheisel et al. 2005; Ling et al. 2003; McMahon and Wall 1985; Mense and Craig 1983) have addressed the function of group III and IV muscle afferents, some of the basic anatomical and physiological properties of these fibers remain unknown. For example, although there have been several studies analyzing the function of group III and IV muscle afferents, most of these studies have largely been limited to fibers that are mechanically sensitive (Chen et al. 2010; Hoheisel et al. 2004, 2005; Xu et al. 2010). However, recent evidence has suggested a significant chemosensitive function of these same afferents (Kaufman and Hayes 2002; Light et al. 2008; Naves and McCleskey 2005). In addition, early physiological studies by McMahon and Wall (1985) suggested that unmyelinated fibers innervating muscle project to deeper laminae of the spinal cord (SC) than those innervating skin. Bulk labeling studies have provided mixed support for these earlier findings (Abrahams 1986; Abrahams and Swett 1986; Craig and Mense 1983). Recently, it has been shown that at least some unidentified unmyelinated muscle afferents do project to the same laminae as cutaneous ones (Ling et al. 2003); however, the belief that they project to different areas of the SC persists (Xu and Brennan 2009).

Current knowledge of the chemosensitive nature of muscle afferent subpopulations also suggests that one group can be thought of as metaboreceptors or ergoreceptors that respond to innocuous levels of metabolites present in muscle after moderate exercise (low lactic acid, ATP, pH 7.0). These afferents are thought to contribute to the activation of the exercise pressor reflex or possibly the sensation of fatigue (e.g., Kaufman and Hayes 2002; Light et al. 2008). A second population of afferents, however, are thought to be responsive to higher levels of lactate and ATP (pH 6.6), which have been shown to be present in muscle during ischemic contractions (Bangsbo et al. 1993, 1996; Hellsten et al. 1998; Li et al. 2003, 2005; Light et al. 2008; Liu et al. 2007; Mohr et al. 2007; Pan et al. 1999; Sinoway et al. 1989; Street et al. 2001; Yagi et al. 2006), and are more nociceptive in function. Here we have employed a novel ex vivo forepaw muscle, median and ulnar nerve, dorsal root ganglion (DRG), SC preparation to determine whether the earlier in vitro findings of Light and colleagues (2008) are validated when the metabolite mixtures are applied directly to the muscles. This preparation will also allow us to further examine the anatomical, neurochemical, and physiological properties of group III and IV muscle afferent fibers, thus defining the different functional groups of afferents innervating muscle.

MATERIALS AND METHODS

Animals. Experiments were conducted with adult (4–6 wk) male Swiss Webster mice (Hilltop Farms, Scottsdale, PA). All animals were housed in group cages, maintained on a 12:12-h light-dark cycle with a temperature-controlled environment, and given food and water ad libitum. All procedures were performed in accordance with National Institutes of Health-approved policies for use of animals in laboratory research and IACUC-approved protocols.
Ex vivo preparation. Mice were anesthetized with a intramuscular hindlimb injection of ketamine and xylazine (90 and 10 mg/kg, respectively) and perfused transcardially with oxygenated (95% O2-5% CO2) artificial cerebrospinal fluid (aCSF; in mM: 127.0 NaCl, 1.9 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26.0 NaHCO3, and 10.0 D-glucose) at 12–15°C. The SC and the right forelimb were dissected in continuity with the median and ulnar nerve, along with the forelimb muscles they innervate (with bone left intact) were dissected in continuity with the median and ulnar nerves well proximal to the muscles to be examined in these studies. After dissection, the preparation was transferred to a separate recording chamber containing chilled oxygenated aCSF. The forepaw was pinned on an elevated platform, keeping the entire paw perfused in a chamber isolated from DRGs and SC. The platform served to provide stability during applied thermal and mechanical stimuli. The bath was then slowly warmed to 31°C before recording.

Recording and stimulation. All recordings were made from the C7, C8, and T1 DRGs as these are the primary source of muscle afferent fibers contained in the median and ulnar nerves. Sensory neuron somata were impaled with quartz microelectrodes (impedance >150 MΩ) containing 15% Neurobiotin (Vector Laboratories, Burlingame, CA) in 1 M potassium acetate. Orthograde electrical search stimuli were delivered through a suction electrode on the nerve to locate sensory neuron somata innervating the muscles. The latency from the onset of this stimulus and the conduction distance between the DRG and the stimulation site were used to calculate the conduction velocity (CV) of the fibers. Group IV afferents were classified as those with a CV < 1.2 m/s, and group III afferents were those with CVs between 1.2 and 14 m/s. Peripheral receptive fields (RFs) were localized by electrically stimulating the muscles with a concentric bipolar electrode. Driven cells with RFs in the muscles then underwent mechanical, thermal, and chemical testing. Mechanical response characteristics were assessed with an increasing series of von Frey hairs ranging from 1 g to 4 g (with diameters of 0.23–0.36 mm). Thermal responses were determined by applying hot (~52°C) or cold (~0°C) saline directly to the paw muscles at the mechanically or electrically determined RF. Each application lasted ~4–5 s. Next, muscles were exposed to an oxygenated “low”-metabolite mixture (15 mM lactate, 1 mM ATP, pH 7.0) and then to a “high”-metabolite mixture (50 mM lactate, 5 mM ATP, pH 6.6) delivered by a valve controller with an in-line heater to maintain solutions at bath temperature. ATP was added to the mixture immediately prior to delivery of metabolites. These two concentrations of metabolites were based on the previous findings of Light et al. (2008). In this earlier study, Light et al. used the results of previous publications reporting the levels of metabolites present in muscles during either exercise (low) or ischemic contraction (high) (Bangsbo et al. 1993, 1996; Connor et al. 2005; Hellsten et al. 1998; Immke and McCleskey 2001; Li et al. 2005; Liu et al. 2007; Mohr et al. 2007; Pan et al. 1999; Sinoway et al. 1989; Street et al. 2001; Sutherland et al. 2001).

Adequate recovery times (~20–30 s) were employed between stimulations. All elicited responses were recorded digitally for off-line analysis (Spike2 software, Cambridge Electronic Design). After physiological characterization, select cells were labeled by iontophoretic injection of Neurobiotin (1 or 2 cells/DRG). Peripheral CV was then calculated from spike latency and the distance between stimulating and recording electrodes (measured directly along the nerve).

Tissue processing and analysis of recorded cells. Once a sensory neuron was characterized and intracellularly filled with Neurobiotin, the preparation was allowed to incubate for 4–5 h to allow diffusion of Neurobiotin into the SC. Then the SC and DRG containing the injected cell were removed and immersion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). DRGs were fixed for 30 min at room temperature (RT), and SCs were fixed overnight at 4°C. SCs and ganglia were then embedded in 10% gelatin, postfixed in 4% paraformaldehyde, and cryoprotected in 20% sucrose. For DRGs, frozen sections (60 μm) were collected in PB and reacted with fluorescently tagged (FITC) avidin to label Neurobiotin-filled cells (Vector Laboratories). Next, each section was processed for calcitonin gene-related peptide (CGRP, 1,200; Molecular Probes, Eugene, OR), acid-sensing ion channel 3 (ASIC3, 1:1,000; Millipore), transient receptor potential vanilloid type 2 (TRPV2, 1:500; gift from Dr. Michael Caterina, Johns Hopkins University), and/or transient receptor potential vanilloid type 1 (TRPV1, 1:500; Alomone) immunohistochemistry. After incubation in primary antiserum, tissue was washed and incubated in Cy3- or Cy5-conjugated donkey anti-rabbit or donkey anti-goat secondary antiserum (1:200; Jackson Immunoresearch). Distribution of fluorescent staining was determined with an Olympus Fluoview 500 laser scanning confocal microscope (Olympus America). Sequential scanning was performed to prevent bleed-through of the different fluorophores.

For visualization of central projections of functionally identified identified cells, 50-μm serial sections of the SCs were collected in PB. Tissue was then incubated in a 1% hydrogen peroxide solution of 1:1 methanol and PB for 15 min at RT. Tissue was then washed in PB and then in 0.05 M Tris containing 0.06% Triton X-100 (Tris-Triton). Sections were then incubated in avidin-biotin-peroxidase complex containing 0.625% Triton X-100 for 4–6 h at RT. Tissue was again washed in Tris-Triton and then in Tris alone. Tissue was then incubated in 3,3’-diaminobenzidine (DAB) containing nickel chloride as an enhancer. Tissue was washed extensively in Tris and serially mounted onto slides. Images were taken on a Leica fluorescence microscope with bright-field optics and images produced by Adobe Photoshop.

Data analysis. One-way ANOVA tests and post hoc analysis (Tukey) were used to analyze differences in instantaneous frequency along with mechanical thresholds of both group III and IV fibers. This information was sorted by neuronal functional type to examine whether or not certain classes of neurons have coherence with regard to the expression of any of the markers tested. Data were also sorted by modality responsiveness and analyzed. Differences in fiber prevalence were determined by Fisher’s exact analysis. P values were set at <0.05.

RESULTS

We have developed a novel mouse forepaw muscle, median and ulnar nerve, DRG, SC recording preparation to comprehensively phenotype individual populations of sensory neurons (Fig. 1). A total of 66 primary muscle afferents were physiologically characterized in 32 Swiss Webster mice. For this analysis, we focused on unmyelinated group IV and thinly myelinated group III muscle afferents. Group IV afferents were classified as those with a CV < 1.2 m/s (mean = 0.49 ± 0.02 m/s, range 0.21–1.02 m/s; n = 56), and group III afferents were those with CVs between 1.2 and 14 m/s (mean 6.47 ± 1.34 m/s, range 2.24–10.19 m/s; n = 10). These CV groups are similar to that previously seen for cutaneous afferents in mouse in a similar ex vivo preparation (e.g., Jankowski et al. 2009a, 2010; Lawson et al. 2008). All cells including both group III and IV fibers had broad inflicted somal action potentials. The somal spike half-amplitude duration for group IV fibers was 2.5 ± 0.13 ms, and the half-amplitude duration for the group III fibers was 1.3 ± 0.18 ms. There were no statistical differences between sets of fibers responding to the various peripheral stimuli for either CV or somal spike duration in either the group III or IV fibers.

Response properties of group III and IV muscle fibers. After examining the peripheral response properties of these 66 fibers with mechanical, thermal (hot and cold), and chemical (me-
tabolite mixtures, high and low concentrations) stimuli, we found that of the different stimulus modalities similar percent-
ages of group III and IV muscle afferents responded to me-
chanical (34%) and thermal (32%; cold 13%, heat 19%) 
stimuli. However, there were significantly more fibers that 
responded when we exposed the muscles to the metabolite 
mixtures (52%; Fig. 2B, P < 0.05) than responded to thermal 
or mechanical stimuli. These chemosensitive afferents were

approximately equally distributed between those that re-
ponded to the “low” concentration of metabolites (metabore-
ceptors) (49%) and those that responded to the “high” concent-
ration (nociceptors) (51%; not shown). Of the cells responding 
to the lower concentration of metabolites, most (65%) did not 
respond when the higher concentrations of metabolites were 
applied. On occasion, some low-metabolite-sensitive afferents 
responded to both metabolite mixtures (35%) but were consid-
ered here to be metaboreceptors because the mean peak instan-
taneous frequency of the response to the high-metabolite mix-
ture was not different from the response to the low-metabolite 
mixture. Finally, some of the group IV fibers (n = 12) 
activated by electrical (concentric electrode) stimulation of 
the muscle did not respond to any of the five stimuli (18% of all 
fibers).

Within the different CV categories, group III fibers (n = 10) 
were most frequently responsive to mechanical stimuli (70%),
although this was not statistically different from the percentage 
responding to thermal (30%; cold 20%, heat 10%) or chemical 
/metabolites (40%) stimuli (Fig. 3). Of the 44 group IV fibers 
responding to natural stimuli we found that there were signifi-
cantly more chemosensitive afferents (54%) than those re-
ponding to thermal (33%; cold 12%, heat 21%) or mechanical

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Fewer fibers responded of these responding to the low (metaboreceptors) and half to modality, most (18 of 24) responded to metabolites, with half concentration. For group IV fibers only responding to one afferents that only responded to heat or the low metabolite responded to the high-metabolite mixture. We found no group III stimuli while one only responded to cold and one only re-
seven single-modality fibers only responded to mechanical (group III 70%; group IV 63%). Of the group III fibers, five of group IV muscle fibers for the various stimuli (not shown).

Most fibers tested responded to a single stimulus modality (group III 70%; group IV 63%). Of the group III fibers, five of seven single-modality fibers only responded to mechanical stimuli while one only responded to cold and one only responded to the high-metabolite mixture. We found no group III afferents that only responded to heat or the low metabolite concentration. For group IV fibers only responding to one modality, most (18 of 24) responded to metabolites, with half of these responding to the low (metaboreceptors) and half to the high (nociceptors) concentrations. Fewer fibers responded only to mechanical (4 of 24) or thermal (2 of 24; 1 hot, 1 cold) stimuli.

However, a small number of fibers responded to more than one stimulus modality, suggesting the possibility of multiple subtypes of group III and IV muscle afferents in mouse. Since we applied five different stimuli to these fibers, the number of fibers in each subtype (responding to >1 stimulus parameter) is quite low. Given the small numbers, we combine groups III and IV together. We found that 18 cells (3 group III and 15 group IV) responded to two or more stimulus modalities (polymodal). These include three cells that responded to the low concentration of metabolites (met-low) and mechanical stimulation; three cells that responded to the high concentration of metabolites (met-high) and mechanical stimulation; two cells that responded to met-high plus cold stimulation; one cell that responded to met-low and heat; three cells that responded to met-high and heat; one cell that responded to mechanical and cold stimuli; and two cells that responded to mechanical plus heat stimuli. An additional three cells responded to all three modalities: three cells responded to met-low, mechanical, and heat, and one responded to met-high, mechanical, and heat (Table 1).

### Table 1. Quantification of polymodal group III and IV muscle afferents as characterized by ex vivo recording

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No. of Polymodals</th>
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<tbody>
<tr>
<td>Met-Low + Mech</td>
<td>3/18</td>
</tr>
<tr>
<td>Met-High + Mech</td>
<td>3/18</td>
</tr>
<tr>
<td>Met-High + cold</td>
<td>2/18</td>
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<td>Met-Low + heat</td>
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<tr>
<td>Met-High + heat</td>
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<td>Mech + cold</td>
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<td>Mech + heat</td>
<td>2/18</td>
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<tr>
<td>Met-Low + Mech + heat</td>
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</tr>
<tr>
<td>Met-High + Mech + heat</td>
<td>1/18</td>
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Met, metabolite; Mech, mechanical.

(27%; Fig. 3, P < 0.02) stimuli. However, comparing the response properties across CV categories (i.e., group III vs. IV fibers) revealed that there were statistically more mechanically sensitive group III muscle afferents than group IV fibers (70% vs. 27%; Fig. 3, P < 0.01). There was no statistically significant differences in the percentage of thermally (30% vs. 33%; cold 20% vs. 12%, heat 10% vs. 21%) or chemically (40% vs. 54%) sensitive afferents between group III and group IV muscle afferents. The mean peak instantaneous frequencies to these various stimuli were also not different between the modalities either when combining the data from the two cell types or when analyzing the differences between group III and group IV muscle fibers for the various stimuli (not shown).

To determine the neurochemical phenotypes and central anatomy of group III and IV muscle afferents, we intracellularly filled select cells after physiological characterization with Neurobiotin. Eighteen muscle sensory neurons were intracellularly labeled, recovered, and immunohistochemically characterized in addition to the corresponding SC segments. We found no differences in the neurochemical phenotypes of group III and IV muscle afferents; therefore results of immunostaining were combined for ease of presentation. We found that three of five metabolite-sensitive nociceptive muscle afferents (60%) stained positively for heat-transducing channel TRPV1 and three of seven intracellularly filled metabolite nociceptors (43%) were immunoreactive for ASIC3 (Fig. 4, Fig. 5). In contrast, none of the intracellularly stained cells that responded to the low-metabo-

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**Fig. 4.** Example of 2 group III muscle afferents immunostained for acid-sensing ion channel 3 (ASIC3) and transient receptor potential vanilloid types 1 and 2 (TRPV1 and TRPV2). A recovered Neurobiotin-stained (arrows) group III muscle afferent (A; green) that responded to the high-metabolite mixture was found to be immunoreactive for ASIC3 (B; blue) but not TRPV1 (C; red). Another group III muscle fiber (D) intracellularly filled with Neurobiotin (arrows) responded to cold but no other stimuli and was immunoreactive for both ASIC3 (E; blue) and TRPV2 (F; red). This particular afferent had a projection into lamina I of the SC (G) and a collateral projection into lamina V (not shown). **Inset:** higher magnification (×40) of the labeled central projection. Scale bars: 50 μm (A–F), 200 μm (G).
Lite mixture (metaboreceptors) were positive for these markers (Fig. 6). Non-metabolite-sensitive cells such as those that were mechanically sensitive and cold sensitive were found to contain ASIC3 in addition to cells that were metabolite insensitive but mechanically sensitive and heat sensitive. Surprisingly, contrary to our previous studies on cutaneous afferents (Jankowski et al. 2009a, 2010; Lawson et al. 2008), mechanically insensitive metabolite-insensitive but heat-sensitive muscle sensory neurons were not found to contain TRPV1 or ASIC3. However, the one silent fiber recovered was found to be immunopositive for TRPV1. One metabolite nociceptor was also found to be immunoreactive for CGRP, and one mechanically sensitive and metabolite-insensitive cell that responded to cold stimuli stained positively for TRPV2.

The central projections of 12 characterized muscle afferent fibers were analyzed in these studies. Of these, 2 were group III muscle afferents and 10 were group IV afferents. The group III fibers had wide-ranging projections extending over one or two segments rostral and caudal to the level of entry into the SC. They both had extensive projections in lamina I and parts of lamina IIo (Fig. 4), but they also had additional less pronounced projections into laminae IV-V (not shown). One of these group III muscle afferent fibers responded only to cold stimuli, while the other only responded to high metabolite concentrations (Fig. 4). Of the 10 group IV muscle afferents, 6 responded to the high concentration of metabolites, while 3 responded only to the low concentration of metabolites. One of the fibers did not respond to any of the stimuli. We found that all of the group IV fibers projected almost exclusively in laminae I and II of the SC (Fig. 5, Fig. 6). Interestingly, both high (Fig. 5)- and low (Fig. 6)-metabolite responders had projections into both laminae I and II of the superficial dorsal horn. In addition, several of these fibers also supported more diffuse projections in lamina III. The rostro-caudal extent of the projections was limited compared with the extent of the group III fibers, but on average they extended roughly one segment immediately rostral and caudal to the point of entry. The overall density of the projections in the superficial dorsal horn was quite similar to that seen for cutaneous C fibers innervating back skin (Albers et al. 2006). Finally, there were no obvious differences between the central projections of those fibers characterized as metaboreceptors and those determined to be nociceptors.

Fig. 5. Example of 2 group IV “high”-metabolite responsive muscle afferents immunostained for ASIC3 and TRPV1. A recovered Neurobiotin-stained (arrows) group IV muscle afferent (A; green) that responded to the high-metabolite mixture and also to heat stimuli was found to be immunoreactive for both ASIC3 (B; blue) and TRPV1 (C; red). Another example of a recovered group IV muscle fiber (D) intracellulary filled with Neurobiotin (arrows) responded to mechanical deformation of the muscles and the high-metabolite mixture and was immunoreactive for TRPV1 (F; red) but not ASIC3 (E; blue). This muscle afferent had a projection into lamina II of the SC (G). Inset: higher magnification (×40) of the labeled central projection. Scale bars: 50 µm (A–F), 200 µm (G).

Fig. 6. Example of 2 group IV “low”-metabolite responsive muscle afferents immunostained for ASIC3 and TRPV1. A Neurobiotin-stained (arrows) group IV muscle afferent (A; green) that responded to the low-metabolite mixture and no other stimuli was not found to be immunoreactive for either ASIC3 (B; blue) or TRPV1 (C; red). The central termination of this afferent was lamina II (D). Another group IV muscle fiber (E) intracellulary filled with Neurobiotin (arrows) responded to both metabolite mixtures and was also not immunopositive for ASIC3 (F; blue) or TRPV1 (G; red). This low responder, however, projected into lamina I of the SC (H). Inset: higher magnification (×40) of the labeled central projection. Scale bars: 50 µm (A–C, E–G), 200 µm (D and H).

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DISCUSSION

The results of these studies provide novel information on the physiological and anatomical properties of group III and IV muscle afferents. Our data show that most group IV muscle afferents are chemosensitive rather than mechanically or thermally sensitive. Conversely, most group III muscle sensory neurons were mechanically sensitive, which confirms earlier reports (Kaufman and Rybicki 1987). Interestingly, the majority of group III and IV muscle afferents responded to a single stimulus modality. For the group IV fibers in particular, this is quite different from their cutaneous counterparts, where the majority of C fibers are polymodal (e.g., Jankowski et al. 2009a; Lawson et al. 2008). Of the chemosensitive fibers it was also notable that muscle afferents were found to be approximately equally distributed between those that responded to a metabolite mixture found in the muscle during work-related activity (metaboreceptors) and those that responded to a metabolite mixture found in muscles during ischemic contractions (nociceptors; Light et al. 2008 and references within). More importantly, we have found that the central projections of these metaboreceptors and metabo-nociceptors project to the same locations in the superficial dorsal horn.

**Chemosensitivity in group III and IV muscle afferents.** While it has been known for decades that afferents that respond to metabolites produced by contracting muscles can affect sympathetic responses (Alam and Smirk 1937), most studies analyzing sensory fiber responses have determined peripheral RFs of both group III and IV afferents by noxious and nonnoxious mechanical stimulation (Adreani et al. 1997; Hayes et al. 2006; Hoheisel et al. 2004, 2005; Kaufman et al. 2002; Xu et al. 2010), which may incorrectly infer that the majority of muscle afferents respond to mechanical deformation of the muscles. Although these latter studies do not specifically state that muscle afferents are all mechanically sensitive, this notion has been more explicitly challenged and clarified by a few recent studies (Light et al. 2008; Naves and McCleskey 2005) and in the present study. While group III afferents recorded here did show a preference for mechanical stimuli, most group IV fibers were sensitive to a mixture of physiologically relevant concentrations of metabolites and had significantly fewer mechanical responses. We were able to more precisely clarify the differences between the present and earlier studies because, unlike many of the prior studies that employed a mechanical search stimulus to localize peripheral RFs, we have used an electrical stimulus to identify afferents innervating the muscle.

Another contributing factor is that we used a combination of metabolites to experimentally evoke chemosensitive responses rather than varying pH, lactate, or ATP individually. Although it has been postulated that lactic acid, ATP, and protons alone can elicit responses in muscle afferents, whether these metabolites are more physiologically relevant stimuli individually or in combination has often been debated (Bangsbo et al. 1993, 1996; Hellstén et al. 1998; Li et al. 2003, 2005; Light et al. 2008; Liu et al. 2007; Mohr et al. 2007; Pan et al. 1999; Sinoway et al. 1989; Street et al. 2001; Yagi et al. 2006). Recently, however, it has been suggested (Imnike and McCleskey 2001, 2003; Naves and McCleskey 2005) and confirmed in an in vitro study (Light et al. 2008) that muscle afferents respond to combinations of lactate, ATP, and protons at physiological levels. Additionally, previous studies suggested that there could be two populations of chemosensitive muscle afferents: those that respond to moderate pH and those that respond to lower pH (Birdsong et al. 2010; Light et al. 2008). Our data support the notion of two separate chemosensitive populations of muscle afferents: those that are metaboreceptors or ergoreceptors thought to be involved in the general homeostasis of the muscle or the sensation of fatigue and those that may be more nociceptive in nature (Light et al. 2008; Naves and McCleskey 2005). In addition, we found that muscle afferents that were characterized as metaboreceptive were neurochemically distinct from those characterized as nociceptive, since the nociceptors often stained positively for TRPV1 and/or ASIC3 while metaboreceptors did not contain these markers. Given the lack of TRPV1 and ASIC3 content in these fibers, metabolite sensitivity may be provided by the presence of purinergic receptors such as P2X3 or P2Y1.

Finally, it is also possible that our findings differed from those of previous studies because of differences between the muscles examined. Here we are examining fibers innervating small forepaw muscles, while several earlier studies primarily examined fibers innervating large limb muscles (e.g., gastrocnemius; e.g., Hayes et al. 2005; Hoheisel et al. 2005; McCord et al. 2009). In addition, given the size of the exposed small muscles, the metabolite solutions could more easily diffuse through them compared with the larger muscles.

Using our newly developed ex vivo recording preparation, we were also able to characterize the responses of these muscle afferents to multiple stimulus modalities. Although we found that the majority of these muscle afferents responded to a single stimulus modality, several fibers exhibited the ability to respond to more than one modality. Given the number of stimuli (5) used in this study, it is difficult to establish the relative numbers of these different functionally defined subsets of polymodal fibers. One interesting finding was that most thermally sensitive fibers were also chemosensitive. Although the role of thermal stimuli or thermally transducing channels in mediating sympathetic reflexes or nociception in muscle afferents has only been explored in a limited number of studies (e.g., Collins 1991; Gao et al. 2007; Mense 2009), future studies could explore the potential role of these channels in the modulation of response properties during exercise and after injury.

**Role of group III and IV muscle afferents as metaboreceptors vs. metabo-nociceptors.** Earlier reports have suggested that distinct populations of metaboreceptive muscle afferents may not exist (Mense 1996; Mense and Stahnke 1983). This was recently challenged by Light and colleagues (2008) on the basis of evidence that combinations of lactate and ATP at particular pH were necessary to evoke responses in muscle afferents (Naves and McCleskey 2005). Here we have shown two populations of group III and IV muscle afferents that responded to a combination of two different concentrations of lactate and ATP at different pH that have been previously reported to mimic metabolite levels in muscle during exercise or ischemic contraction (Light et al. 2008). Those fibers characterized as metaboreceptors also differed from the metabo-nociceptive fibers as they lacked ASIC3 or TRPV1, which were often found in the metabo-nociceptors. Furthermore, two-thirds of those fibers responding to the lower metabolite levels did not respond to the higher concentrations of metabolites. This would suggest basic differences in the transduction processes in these different fiber types. However, several
groups have shown that the ASIC and TRPV channels are important in mediating aspects of the exercise pressor reflex (Hayes and Kaufman 2002; Li et al. 2008), and these channels were thought to be present in the metaboreceptive muscle afferents. Our data suggest that these channels are not present in the classically defined metaboreceptors. This presents an intriguing question on how the channels may be regulating cardiovascular reflexes if they are not present normally in the afferents thought to be directly involved in the responses. One possible explanation for this is that the expression of these channels is altered under certain conditions of muscle activity and afferents that are thought to be classic nociceptors can respond to more moderate concentrations of metabolites after injuries. This is supported by some previous reports specifically in the context of peripheral ischemia or inflammation in that these channels played a much larger role in regulating sympathetic reflexes (Hayes et al. 2008; Kaufman and Hayes 2002; Liu et al. 2010) and nociceptive behaviors (Gautam et al. 2010; Sluka et al. 2003) under injury conditions.

Another possible explanation is the role of purinergic receptors in metabolite responses. Although we have not found a neurochemical marker for the “low”-metabolite responders, the ATP receptor P2X3 has been suggested to play a role in metaboreceptive responses in muscle afferents mediating the pressor reflex (Kaufman and Hayes 2002; McCord et al. 2010). It will be important in the future to determine whether this receptor is present in metaboreceptors and the role this channel plays in afferent response properties to further determine whether there is indeed a distinct class of group III and IV muscle afferents involved in metaboreception versus nociception. Our data show that both the low- and high-metabolite responders project into superficial laminae of the SC, suggesting that they both could play a role in nociceptive responses under certain conditions. Analyses of the response properties, neurochemical identities, and spinal projections of muscle afferents after various peripheral injuries will be necessary future directions. The anatomical finding that both metaboreceptors and metabo-sensitive nociceptors project to the same location in the superficial dorsal horn also raises questions concerning neuronal circuitry. It will be of interest in the future to determine the degree of convergence or divergence of these different inputs (metaboreceptive vs. metabo-nociceptive) to projection neurons in lamina I.

Conclusions. Our data presented here suggest that there are multiple functionally distinct populations of group III and IV muscle afferents. In particular, two populations of muscle afferents can be characterized by their chemosensitivity and distinct neurochemical identity. Although these putative metaboreceptors and metabo-nociceptors are phenotypically distinct, they both project into the same locations in the superficial dorsal horn of the SC. This suggests that although under normal conditions different muscle afferent subpopulations may be more important in regulating responses to work-related activity compared with stronger muscle contractions, after muscle injury both populations may play a role in sympathetic reflexes and pain behaviors. It will be important in the future to determine how different group III and IV muscle afferent subpopulations may contribute to the development of altered cardiovascular reflexes and/or musculoskeletal pain after muscle tissue damage.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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