Comprehensive Phenotyping of Group III and IV Muscle Afferents in Mouse

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ABSTRACT

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 nerves, 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 (metabonociceptors). 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 I (TRPV1), metaboreceptors responding to the lower concentration levels lacked these receptors. Anatomically, group III muscle afferents were found to have projections into lamina I, IIo and deeper laminae in the spinal cord, while all functional types of group IV muscle afferents projected primarily into both lamina I and II. These results provide novel information about the variety of sensory afferents innervating the muscle and provide insight to the types of fibers that may exhibit plasticity after injuries.

Keywords: DRG, metaboreceptors, nociceptors, spinal cord
INTRODUCTION

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); (Xu et al. 2010); (Sluka et al. 2003)) and inflammation ((Walder et al. 2010); (Gautam et al. 2010); (Sluka and Rasmussen 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); (Ikeuchi 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 1988)) 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 (Hoheisel et al. 2004); (Hoheisel et al. 2005); (Xu et al. 2010); (Chen et al. 2010)). However, recent evidence has suggested a significant chemosensitive function of these same afferents ((Kaufman and Hayes 2002); (Naves and McCleskey 2005); (Light et al. 2008)). In addition, early physiological studies by McMahon and Wall (1985) suggested that unmyelinated fibers innervating muscle projected to deeper laminae of the spinal cord than those innervating skin. Bulk labeling studies have provided mixed support of 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 spinal cord persists ((Xu and Brennan 2009)).

Current knowledge of the chemosensitive nature of muscle afferent subpopulations also suggest that one group can be thought of as metaboreceptors or ergoreceptors that respond to innocuous levels of metabolites present in muscle following moderate exercise (low lactic acid, ATP, pH7.0). 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 has been shown to be present in muscle during ischemic contractions ((Bangsbo et al. 1993), (Bangsbo et al. 1996); (Hellsten et al. 1998); (Li et al. 2003), (Li et al. 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 nerves/DRG/spinal cord preparation to determine whether the earlier in vitro findings of Light and colleagues (2008) are validated when the metabolite mixtures are applied to 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 using adult (4-6 weeks) male Swiss Webster mice (Hilltop Farms, Scottdale, PA). All animals were housed in group cages, maintained in a 12h light-dark cycle with a temperature controlled environment and given food and water ad libitum. All procedures were performed in accordance with US and NIH approved policies for use of animals in laboratory research and institutional IACUC approved practices.

Ex-vivo preparation: Mice were anesthetized with a hindlimb IM injection of ketamine and xylazine (90 and 10 mg/kg, respectively) and perfused transcardially with oxygenated (95% O2-5% CO2) artificial CSF (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 spinal cord and the right forelimb was excised and placed in a bath of the same aCSF. The spinal cord was hemisected and the median and ulnar nerves, along with the forelimb muscle it innervates (with bone left intact) was dissected in continuity with its DRGs (C7, C8 and T1). The cutaneous branches were cut as they branch from the ulnar and median nerves well proximal to the muscles to be examined in these studies. Following 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 an isolated chamber from DRGs and spinal cord. 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 >150MΩ) containing 15% Neurobiotin (Vector Laboratories, Burlingame, CA) in 1M 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 stimulation site were used to calculate the conduction velocity of the fibers. Group IV afferents were classified as those with a conduction velocity (CV) < 1.2 m/s, and group III afferents were those with CVs between 1.2 m/s and 14 m/s. Peripheral receptive fields (RF) 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 using an increasing series of von frey hairs ranging from 1g to 4g 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 receptive field. Each application lasted approximately 4-5 seconds. Next, muscles were exposed to an oxygenated “low” metabolite mixture (15mM lactate, 1mM ATP, pH 7.0), then to a “high” metabolite mixture (50mM lactate, 5mM 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 selected 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 either during exercise (low) and ischemic contraction (high) ((Bangsbo et al. 1993), (Bangsbo et al. 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 (approx. 20-30s) were employed between stimulations. All elicited responses were recorded digitally for offline analysis (Spike2 software, Cambridge Electronic Design). After physiological characterization, select cells were labeled by iontophoretic injection of Neurobiotin (1-2 cells per DRG). Peripheral conduction velocity 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 hours to allow diffusion of NB into the spinal cord (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 CGRP, (1:2000; Molecular Probes, Eugene, OR), ASIC3 (1:1000; Millipore), TRPV2 (1:500; gift from Dr. Michael Caterina) and/or 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 antisera (1:200; Jackson Immunoresearch). Distribution of fluorescent staining was determined using Olympus FluoView™ 500 laser scanning confocal microscope (Olympus America Inc.). Sequential scanning was performed to prevent bleed-through of the different fluorophores.

For visualization of central projections of functionally identified cells, 50μm 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 15min at room temperature (RT). Tissue was then washed in PB and then in 0.05M tris containing 0.06% Triton X-100 (Tris/Triton). Sections were then incubated in avidin-biotin-peroxidase complex (company) containing 0.625% Triton X-100 for 4-6hours 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 using bright-field optics and images produced by Adobe Photoshop.
Data analysis: One-way ANOVA tests and posthoc 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 was also sorted by modality responsiveness and analyzed. Differences in fiber prevalence were determined by Fisher’s Exact analysis. P-values were set at $p < 0.05$. 
RESULTS

We have developed a novel mouse forepaw muscle, median and ulnar nerves, DRG, spinal cord 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 conduction velocity (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 m/s 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), (Jankowski et al. 2010), (Jankowski et al. 2010); (Lawson et al. 2008)). All cells including both group III and IV fibers had broad inflected 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 fiber groups.

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 (metabolite mixtures, high and low concentrations) stimuli, we found that of the different stimulus modalities, similar percentages of group III and IV muscle afferents responded to mechanical (34%) and thermal stimuli (32%)
(cold 13%; heat 19%). However, there were significantly more fibers that responded when we exposed the muscles to the metabolite mixtures (52%; Fig. 2b; p value < 0.05) than those that responded to thermal or mechanical stimuli. These chemosensitive afferents were approximately equally distributed between those that responded to the “low” concentration of metabolites (metaboreceptors) (49%) and those that responded to the “high” concentration (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 considered here to be metaboreceptors because the mean peak instantaneous frequency of the response to the “high” metabolite mixture was not different than the response to the “low” 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 than 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 significantly more chemosensitive afferents (54%) than those responding to thermal (33%; cold (12%); heat (21%)), or mechanical stimuli (27%; Fig. 3; p value < 0.02). 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%,
respectively; Fig. 3; p value < 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).

Most fibers tested responded to a single stimulus modality (group III, 70%; group IV,
63%). Of the group III fibers, 5 of 7 single modality fibers only responded to mechanical
stimuli while 1 only responded to cold and 1 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” concentrations (nociceptors). Fewer fibers
responded only to mechanical (4 of 24) or thermal (2 of 24; 1 hot, 1 cold).

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 have applied five different stimuli to these fibers, the number of fibers
in each subtype (responding to more than one stimulus parameter) is quite low. Given
the small numbers, we will combine group 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: 3 cells that responded to the low concentration of metabolites (met-low) and mechanical stimulation; 3 cells responded to the high concentration of metabolites (met-high) and mechanical; 2 cells responded to met-high plus cold stimulation; 1 cell to met-low and heat; 3 cells to met-high and heat; 1 cell to mechanical and cold stimuli; 2 cells to mechanical plus heat. An additional 3 cells responded to all three modalities: 2 cells responded to met-low, mechanical and heat, and 1 responded to met-high, mechanical and heat (Table 1).

Neurochemical Identity and Central Projections of Group III and IV muscle afferents

In order 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. 18 muscle sensory neurons were intracellularly labeled, recovered and immunohistochemically characterized in addition to the corresponding spinal cord 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 5 metabolite sensitive nociceptive muscle afferents (60%) stained positively for heat transducing channel transient receptor potential vanilloid type I (TRPV1) and three of 7 intracellularly filled metabolite nociceptors (43%) were immunoreactive for acid sensing ion channel 3 (ASIC3) (Figs. 4, 5). In contrast, none of the intracellularly stained cells that responded to the low metabolite mixture (metaboreceptors) were positive for these markers (Fig. 6). Non-metabolite sensitive cells such as those that were mechanically and cold sensitive were found to contain ASIC3 in addition to cells that were metabolite
insensitive but mechanical and heat sensitive. Surprisingly contrary to our previous
studies on cutaneous afferents (Lawson et al. 2008); (Jankowski et al. 2009a),
(Jankowski et al. 2010), 2011), 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 calcitonin gene-
related peptide (CGRP) and one mechanically 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 1-2
segments rostral and caudal to the level of entry into the spinal cord. They both had
extensive projections in laminae I and parts of Ilo (Fig. 4) but they also had additional
less pronounced projections into lamina 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
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 lamina I and II of the spinal cord
(Figs. 5,6). Interestingly, both high (Fig. 5) and low (Fig. 6) metabolite responders had
projections into both lamina I and II of the superficial dorsal horn. In addition, several of
these fibers also supported more diffuse projections in lamina III. The rostrocaudal
extent of the projections was limited compared to 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 backskin ((Albers et al. 2006)).
Finally there were no obvious differences between in the central projections of those
fibers characterized as metaboreceptors and those determined to be nociceptors.
DISCUSSION

The results of these studies provide novel information on the physiological and anatomical properties of group III and IV muscle afferents. Our data shows that the 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 counter parts where the majority of C-fibers are polymodal (e.g.(Lawson et al. 2008); (Jankowski et al. 2009a)). Of the chemosensitive fibers it was also notable that muscle afferents were found to be of 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 receptive fields of both group III and IV afferents by noxious and non-noxious
mechanical stimulation (Adreani et al. 1997); (Hayes et al. 2006); (Hoheisel et al. 2004), (Hoheisel et al. 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 current 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 current and earlier studies because unlike many of the prior studies that employed a mechanical search stimulus to localize peripheral receptive fields, we have used an electrical stimulus to identify afferents innervating the muscle.

Another contributing factor is that we used the combination of metabolites to experimentally evoke chemosensitive responses rather than use 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), (Bangsbo et al. 1996); (Hellsten et al. 1998); (Li et al. 2003), (Li et al. 2005); (Light et al. 2008); (Liu et al. 2007; Mohr 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 (Immke and McCleskey 2001), (Immke and
McCleskey 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 ((Naves and McCleskey 2005); (Light et al. 2008)). 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 due to differences between the muscles examined. Here we are examining fibers innervating small forepaw muscle 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 to 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 chemosensory. Although the role of thermal stimuli or thermally transducing channels in mediating sympathetic reflexes or nociception in muscle afferents has only been explored in 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 following 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) based on evidence that *combinations* of lactate, 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 contained 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 presser 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 are 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 (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 presser reflex ((Kaufman and Hayes 2002); (McCord et al. 2010)). It will be important in the future to determine if 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 shows that both the low and high metabolite responders project into superficial laminae of the spinal cord suggesting that they both could play a role in nociceptive responses under certain conditions. Analysis 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 the 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 suggests 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 spinal cord. This suggests that although under normal conditions, different muscle afferent subpopulations may be more important in regulating responses to work related activity compared to 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
The authors declare no potential conflicts of interest, financial or otherwise.

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**FIGURE LEGENDS**

**Figure 1: Schematic of the ex vivo forepaw muscle recording preparation.** Right forepaw muscles (with bone left intact), median and ulnar nerves, T2-C6 dorsal root ganglia (DRGs) and spinal cord (SC) are dissected in continuity and placed in a specially designed chamber for characterization of the response properties of individual sensory neurons in the DRGs. Oxygenated cerebrospinal fluid (CSF) flows into both the outer chamber housing SC and DRGs (A) and inner chamber containing the forepaw muscles (B). The chamber housing forepaw muscles also has an additional inflow where metabolite mixtures are delivered (B). Intracellular recordings of individual DRG neurons are performed and cells with axons contained in the median or ulnar nerves are determined by suction electrodes placed on the sides of each nerve. If a DRG neuron is found in the median/ulnar nerve, an electrical search stimulus is employed using a concentric electrode to determine the cell’s receptive field (RF) in the muscles (C). After RFs are localized, they are probed with thermal (cold and hot saline; D) or mechanical stimulation (von Frey filaments; E). Then the muscles are exposed to the “low” and “high” metabolite mixtures.

**Figure 2: Combined classification of group III and IV muscle afferents by thermal, mechanical and chemical stimuli.** A: Examples of muscle afferent responses to cold, hot, and mechanical (1g) stimuli and the low metabolite and high metabolite mixtures. B: There were similar numbers of thermally (32%) and mechanically (34%) sensitive fibers. However, there were significantly more fibers that responded to the chemical stimuli (metabolite mixtures; 52%). * p value < 0.05, relative to both thermosensitive and mechanosensitive muscle afferents.

**Figure 3: Individual classification of group III and IV muscle afferents.** There were no statistical differences in the percentage of thermally (cold or heat) or chemically (metabolite) sensitive muscle afferents between the group III and group IV fibers. However, there was significantly more mechanically sensitive group III muscle afferents compared to group IV muscle sensory neurons. *p value < 0.05. Although there were no differences in the percentage of cells that responded to each of these different stimulus modalities within the group III afferents, there were significantly more chemosensitive group IV muscle afferents compared to thermally or mechanically sensitive group IV fibers. **p value < 0.05.
Figure 4: Example of two group III muscle afferents immunostained for ASIC3, 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 ASIC 3 (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, was immunoreactive for both ASIC3 (E; blue) and TRPV2 (F; red). This particular afferent had a projection into lamina I of the spinal cord (G) and a collateral projection into lamina V (not shown). Insert: higher magnification (40x) of the labeled central projection. Scale bar A-F, 50μm; Scale Bar G, 200μm.

Figure 5: Example of two 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 ASIC 3 (B; blue) and TRPV1 (C; red). Another example of a recovered group IV muscle fiber (D) intracellularly filled with neurobiotin (arrows) responded to mechanical deformation of the muscles and the high metabolite mixture, was immunoreactive for TRPV1 (F; red) but not ASIC3 (E; blue). This muscle afferent had a projection into lamina II of the spinal cord (G). Insert: higher magnification (40x) of the labeled central projection. Scale bar A-F, 50μm; Scale Bar G, 200μm.

Figure 6: Example of two 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 ASIC 3 (B; blue) or TRPV1 (C; red). The central termination of this afferent was lamina II (D). Another group IV muscle fiber (E) intracellularly filled with neurobiotin (arrows) responded to both metabolite mixtures, was also not immunopositive for ASIC3 (F; blue) or TRPV1 (G; red). This low responder however projected into lamina I of the spinal cord (H). Insert: higher magnification (40x) of the labeled central projection. Scale bar A-C and E-G, 50μm; Scale Bar D and H, 200μm.
Table 1: Quantification of polymodal group III and IV muscle afferents as characterized by *ex vivo* recording. (*Met* = metabolite; *mech.* = mechanical)

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