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SENSORY NERVE FIBERS CONTAINING CALCITONIN GENE-RELATED PEPTIDE IN GASTROCNEMIUS, LATISSIMUS DORSI AND ERECTOR SPINAE MUSCLES AND THORACOLUMBAR FASCIA IN MICE

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Abstract—Chronic pain is a significant burden and much is attributed to back muscles. Back muscles and their associated fasciae make important and distinct contributions to back pain. Peptidergic nociceptors innervating these structures contribute to central transmission and pain modulation by peripheral and central actions. Plastic changes that augment and prolong pain are exhibited by neurons containing calcitonin gene-related peptide (CGRP) following muscle injury. Subpopulations of neurons containing this peptide have been identified in dorsal root ganglia but the distribution of their fibers in skeletal muscles and associated fasciae has not been fully documented. This study used multiple-labeling immunofluorescence and retrograde axonal tracing to identify dorsal root ganglion cells associated with muscle, and to characterize the distribution and density of their nerve fibers in mouse gastrocnemius and back muscles and in the thoracolumbar fascia. Most nerve fibers in these tissues contained CGRP and two major subpopulations of neurons were found: those containing CGRP and substance P (SP) and those containing CGRP but not SP. Innervation density was three times higher in the thoracolumbar fascia than in muscles of the back. These studies show mouse back and leg muscles are predominantly innervated by neurons containing CGRP, an important modulator of pain signal transmission. There are two distinct populations of neurons containing this peptide and their fibers were three times more densely distributed in the thoracolumbar fascia than back muscles.

Key words: calcitonin gene-related peptide, muscle pain, back pain, thoracolumbar fascia, sensory innervation, immunohistochemistry.

INTRODUCTION

Chronic pain has high prevalence and a high proportion is attributed to the musculoskeletal system, especially back muscles (Johannes et al., 2010). The first stage in the development of muscle pain is activation of nociceptive afferent fibers in muscles, described as thin, unmyelinated or lightly myelinated fibers with medium (group III) or slow (group IV) conduction velocity and free nerve endings, some of which contain peptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) (Stacey, 1969; Mense and Meyer, 1985; Reinert et al., 1998; Hoheisel et al., 2005; Mense, 2010b; Jankowski et al., 2013). Neurons containing one or both peptides have been identified in trigeminal and dorsal root ganglia (DRG) (Lee et al., 1985; Morris et al., 2005). They not only contribute to central transmission but also modulate pain sensitivity by multiple actions, and may contribute to chronic pain by promoting prolonged alterations in the function of peripheral or central nociceptive neurons (Woolf and Salter, 2000).

CGRP and SP can be released by the peripheral or central projections of primary afferent neurons and act synergistically to promote inflammation and nociceptor sensitization (Brain and Williams, 1985; Nakamura-Craig and Gill, 1991). Both peptides have vasodilatory actions and CGRP potentiates plasma extravasation induced by SP (Gamse and Saria, 1985). In the periphery, CGRP and SP contribute to sensitization of peripheral nociceptors by actions that include stimulating production of pro-inflammatory cytokines (Shi et al., 2011). CGRP and SP released centrally contribute to central sensitization of nociception by actions in the dorsal horn of the spinal cord (Seybold, 2009). Intrathecal administration of CGRP in rats does not elicit pain behavior, but prolongs that elicited by SP (Wiesenfeld-Hallin et al., 1984). Likewise, intrathecal administration of both peptides facilitates the pain withdrawal reflex to an exaggerated and more prolonged extent than administration of either peptide alone (Woolf and Wiesenfeld-Hallin, 1986). CGRP enhances SP-mediated transmission in three ways: CGRP can potentiate release of SP, presumably by a presynaptic action (Oku et al., 1987); it can enhance excitability of neurons receiving nociceptive inputs, at least in part by potentiating the actions of SP itself (Biella et al., 1991; Seybold et al., 2003; Sun et al., 2004; Bird et al., 2006); and it can inhibit the degradation of SP, thereby prolonging its action (Le Greves et al., 1985).

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Abbreviations: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; IgG, immunoglobulin G; IR, immunoreactivity; NSE, neuron-specific enolase; PBS, phosphate-buffered saline; PGP, protein gene product; SP, substance P.

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Plastic changes in peptidergic sensory nerves that supply muscle may contribute to chronic muscle pain. Following muscle insult, associated neurons of the trigeminal or DRG demonstrate increased CGRP immunoreactivity (IR), increased mRNA for CGRP, increased CGRP peptide content and increased electrical excitability. These changes have been linked to hyperalgesia, allodynia, sensitization of dorsal horn neurons and increases in their receptive field size (Ambalavanar et al., 2006a,b; Harriott et al., 2006; Dessem et al., 2010; Hoheisel et al., 2011; Miyagi et al., 2011; Hoheisel et al., 2013). In addition, inflamed rat gastrocnemius muscles exhibit increased numbers of nerve endings with IR to both CGRP and SP, and to SP only, due to either proliferation of endings or increased peptide content in existing endings (Reinert et al., 1998).

Fasciae associated with muscle are also innervated by peptidergic sensory fibers, (Yahia et al., 1992; Hoheisel et al., 2011; Tesarz et al., 2011; Barker et al., 2014) and may be an under-recognized source of chronic pain. The thoracolumbar fascia is a major connective tissue structure that covers the deep muscles of the back and attaches many muscles of the trunk to the vertebral column, including the abdominal muscles and powerful muscles such as latissimus dorsi and gluteus maximus that act on the proximal limb segments. Its fibers are orientated in multiple planes (Tesarz et al., 2011) and it can resist high tensile loads (Tesh et al., 1987; Barker et al., 2007, 2014). Experimental studies indicate the thoracolumbar fascia makes a distinct contribution to nociception. Hypertonic saline injected into the thoracolumbar fascia in healthy humans results in pain of greater intensity, more unpleasant quality and spreading over a greater area of the back and lower limb, compared to injection into muscle or subcutaneous tissue (Schilder et al., 2014). This pain was associated with chemical stimulation rather than mechanical distention (Schilder et al., 2014).

In summary, muscle pain involves activation of nociceptors and may be prolonged or augmented by adaptations involving these nerves. Subpopulations of nociceptors have been identified according to characteristics such as peptide content, degree of myelination, conduction velocity and soma size, and correlations between the neurochemistry and function of ganglion neurons associated with muscle have been identified (Jankowski et al., 2013), however descriptions of the distribution of their fibers in back muscles and associated fasciae are limited. Most studies have focused on the gastrocnemius muscle of the calf and few have investigated nerve fibers with IR to CGRP but not SP. Therefore, this study aimed to characterize the distribution of nerve fibers with IR to CGRP, with and without IR to SP, in mouse gastrocnemius and back muscles and the thoracolumbar fascia, and to characterize CGRP-immunoreactive DRG cells associated with muscle.

EXPERIMENTAL PROCEDURES

General procedures

All procedures were performed according to the guidelines established by the National Health and Medical Research Council of Australia for the use of laboratory animals in experimental research, and were approved by the Animal Welfare Committee of Flinders University.

Whole-mount preparations and sections of skeletal muscle and fascia

To visualize the 3D organization of nerve fibers in relation to adjacent structures within samples of skeletal muscle and thoracolumbar fascia, multiple-labeling immunohistochemistry of whole-mount preparations was used according to previously established protocols (Gibbins, 2012). To quantify subpopulations of nerve fibers in different tissues, immunolabeling was performed on samples of muscle and fascia that had been sectioned at 12 μm using a cryostat. C57Bl/6 mice (6–8 weeks old, $n = 4$–8 per group) were killed by overdose of isoflurane (Veterinary Companies Australia NSW, Australia) and heart removal. Samples of the latissimus dorsi, erector spinae, and gastrocnemius muscles and the thoracolumbar fascia were isolated and fixed in Zamboni's fixative (2% formaldehyde and 0.5% picric acid in 0.1 M phosphate buffer, pH 7.0) for 24–72 h at 4 °C. Samples were dehydrated through a graded series of ethanol and cleared in xylene before triple immunolabeling to identify subpopulations of nerve fibers.

Retrograde axonal tracing of fibers innervating gastrocnemius

To identify DRG supplying the gastrocnemius muscle, retrograde tracing was performed. C57Bl/6 mice ($n = 4$) were individually placed in a chamber and anesthesia was induced by 5% isoflurane in air, and then maintained by a nose cone (2% isoflurane in 100% medical oxygen). The left hind limb was shaved, cleaned with 70% ethanol and a 25-G needle was used to create a small skin incision to expose the muscle. A Hamilton syringe was used to inject a cholera toxin subunit B conjugate (CTXb-biotin; 1 mg/mL; Molecular Probes, VIC, Australia, $n = 2$; or CTXb-Alexa Fluor-555; 1 mg/mL; Molecular Probes, $n = 2$). The two different CTXb tracers were used due to ready availability and results were found to be equivalent. Four 5 μL volumes (for a total volume of 20 μL) of CTXb-conjugate was injected into multiple sites of the gastrocnemius. The site was then cleaned with ethanol and sealed with a droplet of liquid Opsite (Smith and Nephew, Hull, England). Mice were allowed to recover for 7 days, then euthanized and gastrocnemius muscles, lumbar spinal cord, and the ipsilateral and contralateral L3–L5 DRGs removed. Spinal cord and DRG samples were fixed in Zamboni's fixative (2% formaldehyde and 0.5% picric acid in 0.1 M phosphate buffer, pH 07.0), processed through dimethyl sulfoxide, embedded in polyethylene glycol and sectioned using a microtome. Muscle samples were fixed in Zamboni's fixative, processed through xylene and sectioned using a cryostat as described above. Sections of gastrocnemius muscle, spinal cord and