Nociceptive Craniofacial Muscle Primary Afferent Neurons Synapse in Both the Rostral and Caudal Brain Stem

Dean Dessem, Masayuki Moritani, and Ranjinidevi Ambalavanar

Department of Biomedical Sciences and Program in Neuroscience, University of Maryland, Baltimore, Maryland

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Dessem D, Moritani M, Ambalavanar R. Nociceptive craniofacial muscle primary afferent neurons synapse in both the rostral and caudal brain stem. J Neurophysiol 98: 214–223, 2007. First published May 9, 2007; doi:10.1152/jn.00990.2006. Limited information is available on muscle afferent neurons with fine fibers despite their presumed participation in musculoskeletal disorders, including temporomandibular disorders. To study these neurons, intracellular recordings were made from the central axons of slowly conducting muscle afferent neurons in anesthetized rats. After intraaxonal impalement, axons were characterized by masseter nerve stimulation, receptive field testing, muscle stretching and intramuscular injection of hypertonic saline. Intracellular recordings were made from 310 axons (conduction velocity: 6.5–60 m/s, mean = 27.3 m/s; following frequency: 27–250 Hz, mean = 110 Hz). No neurons responded to cutaneous palpation or muscle stretching. Some axons (n = 34) were intracelluarly stained with biotinamide. These neurons were classified as group II/III nociceptive mechanoreceptors because their mechanical threshold exceeded 15 mN, and conduction velocities ranged from 12 to 40.2 m/s (mean = 25.3 m/s). Two morphological types were recognized by using an object-based, three-dimensional colocalization methodology to locate synapses. One type (IIIHTMVo-Vc) possessed axon collaterals that emerged along the entire main axon and synapsed in the trigeminal principal sensory nucleus and spinal trigeminal subnuclei oralis (Vo), interpolaris (Vi), and caudalis (Vc). A second type (IIIHTMVo-Vc) possessed axon collaterals that synapsed only in caudal Vo, Vi, and Vc. Our previous studies show that muscle spindle afferent neurons are activated by innocuous stimuli and synapse in the rostral and caudal brain stem; here we demonstrate that nociceptive muscle mechanoreceptor afferent axons also synapse in rostral and caudal brain stem regions. Traditional dogma asserts that the most rostral trigeminal sensory complex exclusively processes innocuous somatosensory information, whereas caudal portions receive nociceptive sensory input; the data reported here do not support this paradigm.

INTRODUCTION

Despite the prevalence of musculoskeletal pain, much less is known about the neural mechanisms mediating muscle pain than cutaneous pain. Currently available information also indicates that nociception from deep tissues, including muscle, differs from that of cutaneous pain (for review, see Mense 1993, 2003; Mense et al. 2001).

Temporomandibular disorders (TMD) and masticatory muscle pain are prevalent and can impair mastication, swallowing, and speech. It is estimated that ≥50% of TMD cases are related to masticatory muscle dysfunction (Stohler 1999). Patients with TMD frequently also have fibromyalgia (Hedenberg-Magnusson et al. 1997; Plesh et al. 1996), leading to the speculation that these conditions may involve common mechanisms of muscle pain (Widmer 1997). Masticatory muscle inflammation not only modulates neuropeptide and receptor expression within the trigeminal ganglion (TG) (Ambalavanar et al. 2005, 2006a) but also produces mechanical allodynia in both the hindlimbs and craniofacial region (Ambalavanar et al. 2006b). These facts promote the idea that nociceptive feedback from the jaw muscles may interact with systemic nociceptive mechanisms and play a role in musculoskeletal disorders involving pain distributed throughout the head, neck, and limbs. Very limited information is available on small-diameter muscle afferent neurons despite their presumed participation in these musculoskeletal disorders. Previous physiological studies in vivo (Cairns et al. 2001–2003) have reported that glutamate evokes activity in a subpopulation of slowly conducting masseter muscle neurons and that this activation varies with sex and is partially mediated via N-methyl-D-aspartate (NMDA) receptors. In vitro studies of TG muscle afferent neurons (Connor et al. 2005) show that these neurons possess a variety of putative nociceptive transducer channels and that their excitability is increased after muscle inflammation (Harriott et al. 2006). Anatomical data on these neurons are limited to anatomical tracing studies from the jaw muscles or their nerves in rodents (Ambalavanar et al. 2003; Arvidsson and Raapanna 1989; Zhang et al. 1991) and cats (Capra and Wax 1989; Nishimori et al. 1986; Shigenaga et al. 1988). Although these studies show that muscle afferent neurons have somata of varying sizes in the TG and central processes in the spinal trigeminal tract, they provide no physiological data or morphological information on individual neurons. In this study, we characterize the physiological properties, axonal morphology, and synaptic distribution of definitively identified TG neurons.

METHODS

Male Sprague Dawley rats (335–445 g, n = 86) were used for all experiments. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 86–23, revised 1985). Rats were initially anesthetized with sodium pentobarbital (30 mg/kg ip). Arterial blood pressure, heart rate, and core-body temperature were then monitored and maintained at normal physiological levels for the duration of the experiment. The masseter nerve was exposed at its entry point into the masseter muscle, and the tips of stainless steel wires were placed around the nerve. The threshold for masseter muscle contraction evoked by stimulation was then determined.

Animals were then placed in a stereotaxic frame, an electromagnetic vibrator was attached to the diastema of the lower jaw, and the skin surrounding the masseter muscle was used to form a bath that
was maintained at 37°C. To gain access to the spinal trigeminal tract, the bone was removed and the dura overlying the brain stem incised. The overlying cerebellum was gently aspirated exposing the dorsal surface of the brain stem, which was then covered with 37°C mineral oil. Animals were ventilated and end-tidal CO₂ was maintained between 3.5 and 4%. Gallamine triethiodide (20 mg/kg) was then administered to induce paralysis. Anesthesia was maintained during paralysis by regular supplements of anesthetic. The level of anesthesia was also checked periodically by allowing paralysis to wear off.

Electrophysiological methods

Sharp microelectrodes were fabricated from either quartz or aluminosilicate glass and filled with 3–12% biotinamide (Neurobiotin, Vector Labs) dissolved in 0.25 M KCl and 0.5 M Tris HCl buffer (pH 7.6). Electrode impedances ranged from 80 to 150 MΩ and were advanced into the brain stem at the level of Vo via a stepping motor (Fig. 1A).

Masseter muscle afferent axons were initially identified after intracellular impalement by masseter nerve stimulation. These axons were characterized by threshold, ability to follow 1- to 500-Hz masseter nerve stimulation, stretching of the masseter muscle (2.5-mm mandibular displacement, 0.4 Hz), and receptive field size (Fig. 1, B–D). The receptive field of each axon was also tested using monofilaments (North Coast Medical), a pin “probe” and broad-tipped forceps. A subset of axons was tested after the injection of hypertonic (9%) saline into the masseter muscle.

Depolarizing current (DC, 1–4 nA) was injected into axons with a stable membrane potential less than −40 mV and discontinued if the membrane potential became more positive than −30 mV. Total injection times ranged from 15 to 70 nA min (current × injection time).

Neuronal latency was measured from stimulus artifact to action potential initiation at suprathreshold nerve stimulation. Conduction velocity was calculated by adding peripheral and central conduction distances and dividing by neuronal latency. Maximum following frequency was defined as the frequency at which nerve stimulation failed to evoke an action potential 50% of the time.

Histochemistry and immunocytochemistry

Animals in which axons were injected with biotinamide were killed and perfused with a vascular rinse. Animals in which axons were processed using diaminobenzidine (DAB) were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4), whereas animals in which axons were visualized using Texas Red were fixed with 4% paraformaldehyde in 0.1M PB (pH 7.4). Brain stems were sectioned (50 –100 μm) in the frontal, sagittal, or horizontal plane. Trigeminal ganglia were removed from the skull and cryoprotected, and frozen sections were cut at 25 μm.

FIG. 1. Schematic diagram of the experimental methodology used for in vivo intraaxonal recording and staining in an anesthetized rat. A: sharp microelectrode (a) was used to impale the central axonal process (b) of a single masseter muscle primary afferent neuron in the brain stem. The intracellular afferent response was then examined during: electrical stimulation of the masseter nerve (c), deformation of the masseter muscle (d) and surrounding region, intramuscular injection of hypertonic saline (e), and stretching of the masseter muscle (f) within its physiological range. Biotinamide was injected into the axon after physiological characterization for subsequent morphological analysis. B–D: representative properties of a HTM masseter muscle afferent neuron. B: location of receptive field (black spot) within the masseter muscle (gray area). C: response of the muscle afferent axon to mechanical stimulation (1765 mN) within the muscle receptive field. D: 4 superimposed sweeps showing electrical activation of this axon at threshold stimulation of the masseter nerve (62T). Asterisk denotes stimulus artifact, arrow points to 2 failures of masseter nerve stimulation to evoke an action potential. Membrane potential for this axon was −47 mV, conduction velocity was 26 M/s.
TABLE 1. Electrophysiological properties of high threshold masseter muscle afferent neurons

<table>
<thead>
<tr>
<th>Property</th>
<th>Total Population</th>
<th>Labeled Population</th>
</tr>
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<tbody>
<tr>
<td>Threshold for masseter nerve stimulation (T)</td>
<td>169</td>
<td>34</td>
</tr>
<tr>
<td>Conduction velocity, m/s</td>
<td>97</td>
<td>2.73 ± 10.9</td>
</tr>
<tr>
<td>Following frequency, Hz</td>
<td>33</td>
<td>116 ± 69</td>
</tr>
<tr>
<td>Axon diameter, μm</td>
<td>29</td>
<td>2.9 ± 0.9</td>
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Values are means ± SD.
to very gently touching or brushing the surface of the masseter muscle were briefly encountered but could not be characterized and thus were not included in this study.

Eleven fibers activated by high-threshold electrical stimulation of the nerve and high mechanical stimulation of the muscle were tested after intramuscular injection of hypertonic saline. Spontaneous activity was evoked by intramuscular hypertonic saline in two masseter muscle afferent axons, and the evoked response was altered by hypertonic saline in an additional two axons. None of the masseter muscle afferent axons responded to muscle stretching. Axons were not tested for sensitivity to muscle contraction.

Morphological characteristics of trigeminal ganglion masseter muscle afferent neurons

Thirty-four axons were intracellularly recorded from in the spinal trigeminal tract, activated by electrical stimulation of the masseter nerve and stained sufficiently that their main axon and axon collaterals with boutons could be visualized within the brain stem. Twenty axons were processed using DAB histochemistry, and 14 were visualized using a fluorescent method. In 31 experiments, a single axon was stained per animal; in one additional animal, three axons were stained. Axon diameter ranged from 1.25 to 5.32 μm (Table 1). Receptive fields were isolated in the masticatory muscles for 26 afferent axons. Receptive field properties of stained axons did not differ from unstained high-threshold masseter muscle afferent axons. All stained axons were activated by 1,765 mN but not 98 mN force. Based on these data, the intraaxonally stained afferent neurons were designated as IIIHTM. The response of two of the six stained axons that were tested was altered by intramuscular infusion of hypertonic saline. None of the stained axons responded to muscle stretching, and none could be activated by palpation of the skin or by mandibular movement.

Thresholds for activation of labeled axons from electrical stimulation of the masseter nerve ranged from 6 to 172 times threshold for activation of trigeminal motoneurons [78 T ± 46 (SD)]. This distribution of thresholds did not differ from that of unlabeled masseter muscle axons (Mann-Whitney rank sum test P = 0.87). Conduction velocities for labeled axons ranged from 12 to 44 M/s (24 ± 7 M/s, SD = 7), which did not differ from the distribution for unlabeled axons (Mann-Whitney rank sum test, P = 0.09). A strong linear relationship was found between axon diameter (AD) and conduction velocity [CV = -4.97 + 10.12 AD; r = 0.98]. Following frequencies for stained axons ranged from 27 to 250 Hz [111 Hz ± 66 (SD)], which did not differ from the distribution for unlabeled axons (Mann-Whitney rank sum test, P = 0.79).

The main axon of all stained neurons was located within the dorsomedial spinal trigeminal tract and exited the brain stem in the sensory root of the trigeminal nerve. Twenty-one stained axons were also recovered within the TG, and in four cases, the cell soma was found within the ganglion. No somata or axons were found in the mesencephalic trigeminal nucleus (Vme) or tract for any HTM masseter muscle afferent axons. Two different morphologies were recognized based on the location of axon collaterals and boutons. One neuronal morphology, designated here as type IIIHTM_Vp-Vc, possesses axon collaterals emerging intermittently from the main axon along its entire length (Figs. 2, and 3C and D, 4, and 5). These collaterals were restricted to regions in close proximity to the main axon in the dorsomedial portion of Vp, and Vo, Vi and laminae I and IV/V of Vc. The second morphological type of TG muscle afferent neuron, termed IIIHTM_Vo-Vc also has its main axon in the dorsomedial spinal trigeminal tract (Figs. 3A and 4). Axon collaterals from IIIHTM_Vo-Vc neurons project only to the caudal portion of Vo, Vi, and laminae I and IV/V of Vc. A few of these neurons had collaterals only in Vi, Vc (Fig. 3B).

Colocalization analysis

Synaptophysin staining consisted of a patchy distribution of discrete particles with distinct volumes where staining was absent. Within the Vmo, regions devoid of synaptophysin staining corresponded to the size and location of motoneurons (Luo and Dessem 1999).

To test the colocalization methodology, muscle spindle afferent axons were intracellularly stained and combined with synaptophysin immunocytochemistry. Spindle afferent axons exhibited increased activity during muscle stretching and axons that course in the trigeminal motor root and tracts of Vme and

FIG. 2. Reconstruction of a IIIHTM_Vp-Vc trigeminal ganglion muscle afferent neuron in sagittal view using camera lucida. This neuron enters the brain stem through the sensory root of the trigeminal nerve. The central axonal process gives off axon collaterals with boutons in dorsomedial portions of the trigeminal principal sensory nucleus (Vp) and the spinal trigeminal subnuclei oralis (Vo), interpolaris (Vi), and caudalis (Vc). Electrical threshold for this afferent was 71T. VII, facial nucleus; LR, lateral reticular nucleus; D, dorsal; R, rostral, scale bar = 1 mm.
Probst. Stained somata were observed in the Vme. Because synaptophysin labeling overlapped with stained spindle afferent boutons, a quantitative methodology (Silver and Stryker 2000) was applied to colocalize synaptophysin within boutons. Prior to colocalization, a point-spread function (psf) was generated to calibrate the confocal microscope and determine the z-axis distribution of signal from small particles. Imaging of 0.17-μm fluorescent beads yielded a psf with a half-width of 1.4 ± 0.1 μm. Based on this psf, optical sections used for colocalization were separated by 1 μm.

Colocalization of synaptophysin within intracellularly labeled boutons (n = 6) was examined within the Vmo of two animals. Based on previous ultrastructural studies of spindle afferent terminals (Luo and Dessem 1999), the minimum synaptic vesicle cluster size for object-based colocalization was set to 0.2 μm. Colocalization analysis yielded colocalization indices ranging from 1.1 to 3.4 (1.7 ± 0.9). To determine the amount of colocalization from random colocalization of synaptophysin with boutons, colocalization was computed using the same bouton with a synaptophysin image from the same depth of an adjacent z-stack (n = 6). Colocalization indices for these shuffled images ranged from 0 to 0.08 (0.04 ± 0.03) and were different from indices generated from boutons and synaptophysin within the same optical section (Krusakal-Wallis one-way ANOVA, P < 0.001).

Examination of TG muscle afferent axons processed for synaptophysin (n = 14) revealed discrete particles of synaptophysin within intracellularly stained boutons comparable with that observed in spindle boutons (Fig. 6). Colocalization indices for neurons from five animals (3 IIIHTMv-Vo, 2 IIIHTMVo-Vc) ranged from 0.9 to 3.8 (1.8 ± 0.8) and were significantly higher than indices from random shuffled sections (Kruskal-Wallis one-way ANOVA, P < 0.001). Post hoc testing using Dunn’s method revealed that colocalization indices for IIIHTMv-Vc as well as IIIHTMVo-Vc boutons were higher than indices from randomly shuffled sections (Fig. 7).

**DISCUSSION**

This study applied an object-based, quantitative three-dimensional colocalization methodology to physiologically characterized axon terminals. Using this stringent method, we demonstrate the novel finding that deep tissue primary afferent neurons conveying nociceptive feedback synapse not only in...
caudal regions of the brain stem such as the spinal trigeminal subnuclei interpolaris and caudalis but also in rostral brain stem regions.

Several features demonstrate that the neurons in this study are primary afferent neurons. First, all axons were recorded in a region of the spinal trigeminal tract devoid of interneurons. In addition, responses evoked from masseter nerve stimulation exhibited a constant latency with no evidence of synaptic jitter or spontaneous postsynaptic potentials. Further, a single receptive field was found for each neuron. Finally, when biotinamide was injected, an axon was visualized within the spinal trigeminal tract, sensory root of the trigeminal nerve and in most cases, within the TG. Only a few somata were recovered in the TG probably because of the difficulty in filling a soma from the injection site.

Mechano-nociceptor muscle afferent axons in this study were, however, readily distinguished from Vme afferent axons, which are activated by low-threshold nerve stimulation, respond to muscle stretching, and the axons of which enter the brain stem in the trigeminal motor root, ascend in the Vme tract, and bifurcate to descend in the tract of Probst.

Neuronal recordings were identified as masticatory muscle afferent neurons based on the following. First, all axons re-
sponded to masseter nerve stimulation. No neurons responded to probing or pinching the skin. The possibility that neurons were jaw joint afferent neurons was eliminated because none responded to jaw movement or probing the jaw joint region. Finally a receptive field was localized within the masseter muscle for many neurons. Most of the axons in this study can be classified as group III based on conduction velocity and axon diameter (Mense et al. 1985). A small proportion of high-threshold axons possessed conduction velocities > 30 m/s and thus would be classified as group II. Previous studies have reported that some high-threshold mechanoreceptors from deep tissues also have group II conduction velocities (Hoheisel et al. 1989). No axons in this study were considered to be secondary muscle spindle afferent neurons because they did not exhibit the regular, spontaneous discharge or stretch sensitivity characteristic of secondaries (Dessem et al. 1997) and their axons were located more laterally in the brain stem than spindle axons (Luo et al. 1995). Recordings were occasionally made from axons with much slower conduction velocity and dramatically longer duration action potentials than the majority of axons. These correspond to in vitro recordings (Harriott et al. 2006) and presumably are from group IV neurons.

Neurons that did not respond to 98 mN (357 g/cm²) force but responded to 1,765 mN (2,115 g/cm²) force applied to the muscle were classified as high-threshold mechanoreceptors. These values are similar to that reported to activate hindlimb and masseter muscle high-threshold mechanoreceptors in vivo (Cairns et al. 2002; Paintal 1960) Muscle mechanoreceptors in this study thus were considered to be noxious mechanoreceptors because they were not activated by innocuous stimulation (stroking, brushing, stretch within the physiological range) and their mechanical thresholds are comparable to previously reported noxious muscle mechanoreceptors.

Previous studies report the presence of low-threshold mechanoreceptors in muscle (Kaufman et al. 1983; Mense and Meyer 1985; Paintal 1960). Very few low-threshold mechanoreceptors were encountered in this study, and none were fully characterized. Many low-threshold nonspindle, muscle mechanoreceptors are stretch sensitive (Cleland et al. 1990) and thus differ from the stretch-insensitive, high-threshold mechanoreceptors described here.

Muscle HTM neurons in this study exhibited no spontaneous activity and thus were not sensitized. This is consistent with most studies of muscle mechano-noceptrors (Abrahams et al. 1984; Ge and Khalsa 2003; Khalsa and Ge 2004; Marchettini et al. 1996; Sandercock 2004; Simone et al. 1994) although some report a subpopulation of slowly conducting muscle afferent neurons with spontaneous activity (Cairns et al. 2001; Kumazawa and Mizumura 1977). Receptive fields for neurons in this study were small, localized within muscle, and showed no signs of convergence from other tissues. Previous studies also report that high-threshold muscle mechanoreceptors possess relatively small receptive fields (Abrahams et al. 1984; Ge and Khalsa 2003; Kumazawa and Mizumura 1977; Sandercock 2004). No neurons exhibited multiple receptive fields as reported for some hindlimb muscle afferent neurons (Kumazawa and Mizumura 1977; Mense and Meyer 1985).

Muscle mechanoreceptors in this study rapidly adapted to mechanical stimulation. This is consistent with hindlimb high-threshold muscle mechanoreceptors (Kumazawa and Mizumura 1977; Paintal 1960). In contrast, Cairns et al. (2002, 2003) reported that group III masseter muscle afferent neurons slowly adapt to mechanical stimulation. Cairns and coworkers stimulated neurons by probing through the skin and the differing response properties therefore may be due to differences in stress transmitted to the sensory receptor when the skin is present. Consistent with this hypothesis is the fact that slowly conducting human muscle afferent neurons adapt slowly when probed through the skin (Marchetti et al. 1996; Simone et al. 1994). Slowly adapting muscle mechanoreceptors have also been reported in vitro (Ge and Khalsa 2003; Taguchi et al. 2005). Muscle deformation in these studies likely differed from in vivo because mechanosensitivity was tested while the muscle rested on a rigid platform.

Less than half of the muscle mechanoreceptor neurons in this study exhibited altered response properties following intramuscular infusion of hypertonic saline. Given that hypertonic saline increases the spontaneous activity of both high- and low-threshold mechanoreceptors (Cairns et al. 2003; Kumazawa and Mizumura 1977; Paintal 1960) and perhaps all muscle afferent neurons (Iggo 1960), the low percentage of neurons that responded to hypertonic saline may be a methodological artifact.

One half of the axons in this study were not capable of following 100-Hz nerve stimulation. Some (Amano et al. 1986; Ro and Capra 1999) have utilized the ability to follow frequencies > 100–500 Hz as a criterion to identify primary afferent neurons. This study demonstrates that using high-frequency following to identify small-diameter, primary afferent neurons is problematic.

Central afferent axonal projections

Masseter muscle noxious mechanoreceptors in this study exhibited two distinctly different axonal morphologies. One type, IIIHTMVp-Vc, synapsed throughout the dorsomedial portion of the entire trigeminal brain stem sensory complex. A second type, IIIHTMVo-Vc, projected to the dorsomedial portions of the caudal brain stem. All axons were impaled with microelectrodes located in the rostral portion of Vo. The lack of axon collaterals in the rostral part of Vo and in Vp observed in type IIIHTMVo-Vc axons therefore cannot be attributed to insufficient staining because regions absent axon collaterals were closer to the injection site than regions with stained axons. In spite of the two distinctly different axonal morphologies, no differences were detected in the physiological properties of each morphological mechanoreceptor type. High-threshold muscle mechanoreceptive neurons in hindlimb muscles also display two axonal trajectories in the spinal cord yet have indistinguishable response properties (Hoheisel et al. 1989; Mense and Prabhaker 1986).

Intracranial staining and colocalization in this study demonstrate that IIIHTMVp-Vc and IIIHTMVo-Vc neurons synapse in laminae I and IV/V of Vc. These synapses may be onto trigeminalthalamc and/or trigemino-trigeminal neurons located in these laminae (Dado and Giesler 1990; Jacquin et al. 1990; Luo and Dessem 1995; Shigenaga et al. 1979). Previous studies report that fine masseter muscle afferent neurons project to Vc. Tracing studies in which horseradish peroxidase was applied to the masseter nerve report terminal labeling in laminae I and V of Vc (Ardivsson and Raapannpa 1989; Nishimori et al. 1986; Shigenaga et al. 1988). Capra and Wax (1989) not only provide
evidence that masseter muscle afferent neurons project to Vc but that their cell bodies are located within the TG. Although these studies provide evidence that some masseter muscle afferent neurons project to Vc, they provide no information about their modality. Cairns and co-workers (2001, 2002) show that some of these muscle afferent neurons are group III although their methodology cannot establish that these neurons synapse in Vc. Additional evidence that muscle afferent neurons project to Vc comes from studies that describe neurons in lamina I and laminae IV—VI, which are activated by noxious stimulation of the masticatory muscles (Hu et al. 1992; Kojima 1990; Sessle et al. 1986). In the spinal cord, muscle afferent neurons project primarily to laminae I, II, V, and VI with a sparse distribution to III and IV (Panneton et al. 2005). In limb muscles, group III high-threshold muscle mechanoreceptors project to laminae I and V (Hoheisel et al. 1989) whereas group IV synapse in laminae I and II (Ling et al. 2003). Thus the group III TG mechano-nociceptors described here have a distribution comparable to hindlimb thinly myelinated muscle afferent axons.

Masseter afferent axons in this study synapsed in Vi. Previous studies (Ardivsson and Raappana 1989; Nishimori et al. 1986; Shigenaga et al. 1989) describe terminal labeling in Vi following the application of neuroanatomical tracer to the masseter nerve. This labeling was confined to the region immediately adjacent to the spinal trigeminal nucleus and thus corresponds to the area where synapses were found in this study. Additional anatomical evidence for a projection of TG masseter muscle afferent neurons to Vc comes from double-labeling studies (Capra and Wax 1989; Wang et al. 2006). Electrophysiological studies describe neurons in Vi that respond to noxious stimulation of the masseter muscle (Hayashi et al. 1984; Ohya 1992; Ohya et al. 1993; Ro and Capra 1999) and thus may be postsynaptic to the axons described here. These interneurons project to the contralateral ventroposteromedial nucleus of the thalamus and the cerebellum (Ohya et al. 1993).

One type (IIIHTM_Vp-Vc) of mechano-nociceptor in this study projected to the entire Vc. This location is consistent with rat tracing studies (Ardivsson and Raappana 1989; Zhang et al. 1991). In contrast to these rodent studies, tracing studies in cats have not reported this projection (Capra and Wax 1989; Shigenaga et al. 1988). Evidence that slowly conducting muscle afferent neurons project to Vc comes from studies showing that Vc neurons are facilitated by injecting mustard oil into the masseter muscle (Hu et al. 1992). Although few neurons in the dorsomedial Vc project to the thalamus (Luo and Dessem 1995), many project to the Vmo (Luo et al. 2001; Travers and Norgren 1983; Vornov and Sutin 1983), and thus afferent neurons projecting to this region may be involved in noxious motor responses (Svensson et al. 2000; Westberg et al. 1997).

This study demonstrates a previously unrecognized nociceptive projection from the masseter muscle to the Vp. Although the spatial extent of this projection is quite restricted, the synaptic density is high, suggesting that a small number of neurons receive strong nociceptive synaptic input. Previous labeling studies (Ardivsson and Raappana 1989; Shigenaga et al. 1988) from the masseter nerve mention that primary afferent fibers project to the dorsomedial Vp but do not speculate on their function. Indirect evidence that small-diameter afferent neurons synapse in Vp comes from the finding that c-fos expression can be induced in dorsal Vp neurons within a few hours after high-intensity stimulation of the TG (Takemura et al. 2000). Eisenman and co-workers (1963) also report nociceptive neurons in the dorsomedial Vp although they were not able to determine whether these neurons receive direct input from HTM muscle afferent neurons. This study demonstrates for the first time that Vp receives direct input from deep tissue nociceptive primary afferent neurons. Although the projection of neurons postsynaptic to this input is unknown, most neurons in the dorsomedial portion of Vp project to the thalamus (Fukushima and Kerr 1979; Luo and Dessem 1999).

**Colocalization**

We applied an object-based three-dimensional colocalization methodology to axon terminals. Previous studies have used confocal methods either to study physiologically uncharacterized axons (Silver and Stryker 1999; Wouterlood et al. 2003) or have used a qualitative methodology (Lamotte d’Incamps et al. 1998). The potential for false colocalization in this study was greatly reduced during image capture by using thin optical sections, not oversaturating pixels, and including only synaptophysin pixels with their highest intensity in the optical plane examined for colocalization. Additionally, an object-based colocalization methodology was utilized that required 100% overlap between synaptophysin clusters and axon terminals (Silver and Stryker 2000).

The specificity of the colocalization method employed here was tested by staining muscle spindle afferent axons and determining whether synaptophysin was colocalized in stained boutons. The physiology and axonal trajectory of these axons was consistent with spindle afferent axons (Dessem et al. 1997). The colocalization methodology demonstrated that colocalization indices from spindle afferent boutons are dramatically higher than those generated from random. Previous ultrastructural studies (Luo and Dessem 1999) indicate that the number and location of synapses exceeds the number of axonal swellings. Because the methodology applied here depends on the location of synaptic vesicle clusters and not axonal morphology, it provides a more accurate means of determining the density and location of synapses at the light microscopical level than counting terminal and en passant axonal swellings.

**Models of brain stem nociceptive circuitry**

Although textbook accounts of orofacial pain perpetuate the notion that craniofacial nociceptive afferent neurons synapse exclusively in Vc, considerable evidence has established that Vc is involved in orofacial nociception (Dalle et al. 1999; Duale et al. 2001; Pajot et al. 2000; Parada et al. 1997; for review see Sessle 2000). Most nociceptive models that incorporate Vc, however, emphasize inputs relayed from the caudal brain stem (Woda 2003). This study demonstrates that nociceptive primary afferent axons synapse in Vc and thus provide nociceptive feedback to Vc directly from muscle without admixing with other sensory inputs. Recent studies have also identified the transition region between Vc and Vp as an important area in craniofacial nociception (Bereiter 2001; Wang et al. 2006). This study demonstrates that group III muscle nociceptive afferent neurons synapse only in the dorsomedial region immediately adjacent to the trigeminal tract at the dorsal surface.
Inflammation of craniofacial

Dorsomedial portion of the brain stem.

Feedback via masticatory muscle afferent synapses in both rostral and caudal brain stem regions (Luo et al. 1995), suggesting that these synapses constitute only a portion of a more extensive nociceptive afferent termination. Long-standing emphasis has been placed on rostral regions of the trigeminal sensory complex processing innocuous somatosensory information and caudal brain stem regions conveying nociceptive mechanisms. Previous studies demonstrate that low-threshold masticatory muscle mechanoreceptors synapse in both rostral and caudal brain stem regions (Luo et al. 1995), whereas this study demonstrates that nociceptive muscle mechanoreceptors synapse not only in Vc but throughout the entire spinal trigeminal sensory nucleus and Vp. Taken together these findings indicate that feedback via masticatory muscle afferent groups I–III does not readily fit into a rostral innocuous/caudal nociceptive paradigm but rather that both innocuous and nocuous muscle afferent feedback is distributed along the entire dorso-medial portion of the brain stem.

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