Nociceptive Craniofacial Muscle Primary Afferent Neurons Synapse in both the Rostral and Caudal Brainstem

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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$^\text{M/s}$, mean=27.3$^\text{M/s}$; following frequency 27-250Hz, mean=110Hz). No neurons responded to cutaneous palpation or muscle stretching. Some axons (n=34) were intracellularly stained with biotinamide. These neurons were classified as group II/III noxious mechanoreceptors since their mechanical threshold exceeded 15mN and conduction velocities ranged from 12-40.2$^\text{M/s}$ (mean=25.3$^\text{M/s}$). Two morphological types were recognized by utilizing an object-based, three-dimensional colocalization methodology to locate synapses. One type (IIIHTM$_{Vo-Vc}$) possessed axon collaterals which 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 (IIIHTM$_{Vo-Vc}$) possessed axon collaterals which 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 brainstem; here we demonstrate that nociceptive muscle mechanoreceptor afferent axons also synapse in rostral and caudal brainstem regions. Traditional dogma asserts that the most rostral trigeminal sensory complex exclusively processes innocuous somatosensory information while caudal portions receive nociceptive sensory input, the data reported here do not support this paradigm.

Key words: pain, confocal microscopy, trigeminal, musculoskeletal, temporomandibular disorders
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; Mense et al. 2001; Mense 2003).

Temporomandibular disorders (TMD) and masticatory muscle pain are prevalent and can impair mastication, swallowing and speech. It is estimated that at least 50% of TMD cases are related to masticatory muscle dysfunction (Stohler 1999). Patients with TMD frequently also have fibromyalgia (Plesh et al. 1996; Hedenberg-Magnusson et al. 1997) 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, 2002, 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 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 following 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 (Arvidsson and Raappana 1989; Zhang et al. 1991; Ambalavanar et al. 2003) and cats (Nishimori et al. 1986; Shigenaga et al. 1988; Capra and Wax 1989). While 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.

**Materials and Methods**

Male, Sprague Dawley rats (335-445g, n=86) were used for all experiments. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985). Rats were initially anesthetized with sodium pentobarbital (30mg/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 which was maintained at 37°C. To gain access to the spinal trigeminal tract, the bone was removed and the dura overlying the brainstem incised. The overlying cerebellum was
gently aspirated exposing the dorsal surface of the brainstem which was then covered with 37°C mineral oil. Animals were ventilated and end-tidal CO₂ was maintained between 3.5-4%. Gallamine triethiodide (20mg/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 brainstem 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 1) threshold, 2) ability to follow 1-500Hz masseter nerve stimulation, 3) stretching of the masseter muscle (2.5mm mandibular displacement, 0.4Hz), and receptive field size (Fig. 1B-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 following the injection of hypertonic (9%) saline into the masseter muscle.

Depolarizing current (DC, 1-4nA) was injected into axons with a stable membrane potential less than -40mV and discontinued if the membrane potential became more positive than -30mV. Total injection times ranged from 15 to 70nA minutes (current x 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 diaminobenzidene (DAB) were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4) while animals in which axons were visualized using Texas Red were fixed with 4% paraformaldehyde in 0.1M PB (pH 7.4). Brainstems were sectioned (50-100μm) in either the frontal, sagittal or horizontal plane. Trigeminal ganglia were removed from the skull, cryoprotected and frozen sections were cut at 25μm.

Processing for DAB consisted of placing sections in 1-2% normal goat serum (Vector S-100) and 1% Triton X-100 in 0.01M PBS followed by incubation in avidin biotin complex (1:50 Elite Vectastain; Vector). Tissue sections were then reacted using nickel-DAB with H₂O₂. Processing axons for Texas Red consisted of incubation in avidin-biotin complex (1:50) and incubation in 4% Texas Red avidin DCS (A-206, Vector). Sections were then either counterstained with a fluorescent Nissl stain (NeuroTrace, Molecular Probes) or processed for synaptophysin. Synaptophysin staining consisted of incubation in mouse anti-synaptophysin antibody (1:10,000, MAB5258 Chemicon) followed by anti-mouse FITC (1:400). To evaluate
nonspecific labeling some sections were incubated without either the primary or secondary antibody. No evidence of positive immunoreactivity was found in these cases.

**Morphological analysis**

The morphology of well-stained axons were reconstructed using either a camera lucida or a reconstruction system (Capowski and Sedivec 1981 or Neurolucida (MicroBrightField). Brainstem landmarks were visualized after counterstaining with either neutral red or fluorescent Nissl stain. The atlas of Paxinos and Watson (1986) was used as a reference for brainstem anatomy.

**Confocal microscopy and colocalization**

Synapses were identified by determining sites where synaptophysin was colocalized within an intracellularly stained axon. This methodology was initially tested by colocalizing synaptophysin within boutons of muscle spindle afferent fibers in the trigeminal motor nucleus (Vmo) (Luo and Dessem 1999; Luo et al. 2001). Spindle afferent axons were physiologically identified (Dessem et al. 1997) and stained with biotinamide (n=5) in two animals.

A point-spread function (psf) was generated for a Zeiss 510Meta confocal microscope by imaging $0.175 \pm 0.0005\mu m$ beads (Molecular Probes, P-7220) at $0.5\mu m$ intervals. Beads were imaged using a 63x objective (1.4NA) (zoom factor=4, pinhole=106μm (1 Airy unit)) resulting in a pixel size of 0.11μm. Images were averaged (2 scans) and thresholded using NIH Image software to retain pixels with a value greater than two (scale 0-255). The number of optical sections in which each bead could be observed and the mean pixel intensity in each section were
then used to generate a psf.

A three-dimensional object-based colocalization methodology was employed to examine the colocalization of synaptophysin within labeled axons. Image stacks consisting of 5-7 optical sections were collected at 0.5μm intervals using the same parameters used to generate the psf except that sequential line scanning (Zeiss multitrack) was employed so that both FITC and Texas Red images could be collected without repositioning the z-axis. Colocalization analysis was performed using the methodology found at (http://phy.ucsf.edu/~idl/colocalization.htm) and in Silver and Stryker (2000). Briefly this technique consisted of isolating the neuropil and standardizing signal intensity between sections. Axon segments were then traced and compared to segmented synaptophysin clusters. For a presynaptic site to be considered colocalized it had to have its greatest intensity within the optical section examined for colocalization, be located within the labeled axon segment and be 0.2μm or larger. The colocalization index developed by Silver and Stryker (2000) was employed:

\[
\text{colocalization index} = \frac{p}{(a)[s(t/n)]}
\]

where \(p\) is the total summed intensities of synaptophysin within colocalized presynaptic sites, \(a\) is the number of pixels within intracellularly stained axon segments localized to the optical section examined for colocalization, \(s\) is the average pixel intensity of synaptophysin, \(t\) is the total number of pixels in the image, \(n\) is the total number of neuropil pixels in the image.

**Results**
Physiological characteristics of muscle afferent neurons

Electrophysiological recordings were conducted within the spinal trigeminal tract in 60 rats. Intraaxonal recordings were made in 310 axons which were activated by electrical stimulation of the masseter nerve. Single action potentials were evoked not only by threshold stimulation of the masseter nerve but also by suprathreshold stimulation (Fig. 1D). The mean threshold for activation of these axons was 88 times greater than the threshold for motoneuron activation while the mean conduction velocity was 27.3 m/s and the mean following frequency was 116 Hz (Table 1). Post-mortem dissection following each experiment confirmed that the stimulating wires encircled the masseter nerve just prior to entry into the masseter muscle. Most muscle afferent action potentials exhibited no prominent deflection on the falling phase (Fig. 1D). In a few instances recordings were made in axons activated by masseter nerve stimulation with substantially longer duration action potentials, longer latencies and prominent deflections on the falling phase of the action potential.

Receptive fields for masseter muscle axons were confined to 3-5mm regions within the belly of the masseter muscle (Fig. 1B). Multiple receptive fields were not found for any of these axons. None of these axons responded to palpation of the skin or to movement of the jaw joint. The mechanical sensitivity of axons was assessed by probing the masseter muscle and surrounding region using monofilaments which generated 98mN and 1765mN of force. Most axons activated by masseter nerve stimulation responded to probing of the masseter muscle with 1765mN force but not with 98mN force (Fig. 1C). The longer latency of the response evoked by mechanical stimulation as compared to electrical stimulation is apparently due primarily to
muscle compliance. A few axons which responded 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 following 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 brainstem. 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 - 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 1765 mN but not 98 mN of force. Based upon 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 6-172 times threshold for activation of trigeminal motoneurons (mean=78T, SD=46). 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 (mean=24 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 27-250Hz (mean=111Hz, SD=66) 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 brainstem 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 upon 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. 2,3C-D,4,5). 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,4). Axon collaterals from IIIHTMVo-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 which coursed in the trigeminal motor root and tracts of Vme and Probst. Stained somata were observed in the Vme. Since 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 0.17μm fluorescent beads yielded a psf with a half-width of 1.4μm±0.1μm. Based upon this psf, optical sections used for colocalization were separated by 1μm.

Colocalization of synaptophysin within intracellularly labeled boutons (n=6) was examined within the Vmo of two animals. Based upon previous ultrastructural studies of spindle afferent terminals (Luo and Dessem, 1999), the minimum synaptic vesicle cluster size for object-based colocalization was set to 0.2μm. Colocalization analysis yielded colocalization indices
ranging from 1.1 to 3.4 (mean=1.7, SD=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, (mean=0.04, SD=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 to that observed in spindle boutons (Fig. 6). Colocalization indices for neurons from 5 animals (3 IIIHTMVp-Vc, 2 IIIHTMVo-Vc) ranged from 0.9 – 3.8 (mean=1.8, SD=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 IIIHTMVp-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 brainstem such as the spinal trigeminal subnuclei interpolaris and caudalis but also in rostral brainstem 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 post-synaptic 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 approximately 7mm 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 whose axons enter the brainstem 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 upon the following. First, all axons responded 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 upon conduction velocity and axon diameter (Mense et al. 1985). A small proportion of high threshold axons possessed conduction velocities greater than 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 since 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 brainstem 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 which did not respond to 98mN (357g/cm²) force but responded to 1765mN (2115g/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 (Paintal, 1960, Cairns et al, 2002) 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 (Paintal 1960; Kaufman et al. 1983; Mense and Meyer 1985). Very few low-threshold mechanoreceptors were encountered in this study and none were fully characterized. Many low-threshold non-spindle, 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-nociceptors (Abrahams et al. 1984; Simone et al. 1994; Marchettini et al. 1996; Ge and Khalsa 2003; Khalsa and Ge 2004; Sandercock 2004) although some report a sub-population of slowly conducting muscle afferent
neurons with spontaneous activity (Kumazawa and Mizumura 1977; Cairns et al. 2001). 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 (Kumazawa and Mizumura 1977, Abrahams et al. 1984; Ge and Khalsa 2003, 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 (Paintal 1960; Kumazawa and Mizumura 1977). 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 (Simone et al. 1994; Marchetti et al. 1996). 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 since 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
(Paintal 1960; Kumazawa and Mizumura 1977; Carins et al. 2003) 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 100Hz nerve stimulation. Some (Amano et al. 1986; Ro and Capra 1999) have utilized the ability to follow frequencies greater than 100-500Hz 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 IIIHTM_{Vp-Vc} synapsed throughout the dorsomedial portion of the entire trigeminal brainstem sensory complex. A second type IIIHTM_{Vo-Vc} projected to the dorsomedial portions of the caudal brainstem. 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 IIIHTM_{Vo-Vc} axons therefore cannot be attributed to insufficient staining since 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 (Mense and Prabhakar 1986; Hoheisel et al. 1989).
Intracellular staining and colocalization in this study demonstrate that IIIHTM Yö-Vc and IIIHTM Yö-Vc neurons synapse in laminae I and IV/V of Vc. These synapses may be onto trigeminothalamic and/or trigemino-trigemino neurons located in these laminae (Shigenaga et al. 1979; Dado and Giesler 1990; Jacquin et al. 1990; Luo and Dessem 1995). 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 (Nishimori et al. 1986; Shigenaga et al. 1988; Ardvisson and Raappana 1989). 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. While 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, V, and VI which are activated by noxious stimulation of the masticatory muscles (Kojima 1990; Sessle et al. 1986; Hu et al. 1992). 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) while 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 (Nishimori et al.
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1986; Shigenaga et al. 1988; Ardvisson and Raappana 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 Vi comes from double-labeling studies (Capra and Wax 1989; Wang et al. 2006). Electrophysiological studies describe neurons in Vi which 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 post-synaptic 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 (IIHTM\textsubscript{Vp-Vc}) of mechano-nociceptor in this study projected to the entire Vo. This location is consistent with rat tracing studies (Ardvisson and Raappana 1989; Zhang et al. 1991). In contrast to these rodent studies, tracing studies in cats have not reported this projection (Shigenaga et al. 1988; Capra and Wax 1989). Evidence that slowly conducting muscle afferent neurons project to Vo comes from studies showing that Vo neurons are facilitated by injecting mustard oil into the masseter muscle (Hu et al. 1992). While few neurons in the dorsomedial Vo project to the thalamus (Luo and Dessem 1995), many project to the Vmo (Travers and Norgren 1983; Vornov and Sutin 1983, Luo et al. 2001) and thus afferent neurons projecting to this region may be involved in noxious motor responses (Westberg et al. 1997; Svensson et al. 2000).

This study demonstrates a previously unrecognized nociceptive projection from the
masseter muscle to the Vp. While 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 (Shigenaga et al. 1988; Ardvisson and Raappana 1989) 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 following 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. While the projection of neurons post-synaptic 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 over saturating pixels and by including only synaptophysin pixels with their highest intensity in the optical plane examined for colocalization. Additionally, an object-based colocalization
methodology was utilized which 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. Since the methodology applied here depends upon 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 brainstem nociceptive circuitry

While textbook accounts of orofacial pain perpetuate the notion that craniofacial nociceptive afferent neurons synapse exclusively in Vc considerable evidence has established that Vo is involved in orofacial nociception (Parada et al. 1997; Dallel et al. 1999; Pajot et al. 2000; Duale et al. 2001; for review see Sessle 2000). Most nociceptive models which incorporate Vo however emphasize inputs relayed from the caudal brainstem (Woda 2003). This study demonstrates that nociceptive primary afferent axons synapse in Vo and thus provide nociceptive feedback to Vo directly from muscle without admixing with other sensory inputs. Recent studies have also identified the transition region between Vi and Vc 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 this level. A novel projection from masticatory muscle nociceptors to a small region of Vp has also been identified. The brainstem distribution of nociceptive muscle afferent synapses demonstrated here overlaps with regions where CGRP-containing trigeminal afferent neurons terminate (Sugimoto et al. 1997) suggesting that these synapses constitute only a portion of a more extensive nociceptive afferent termination. Longstanding emphasis has been placed upon rostral regions of the trigeminal sensory complex processing innocuous somatosensory information and caudal brainstem regions conveying nociceptive mechanisms. Previous studies demonstrate that low-threshold masticatory muscle mechanoreceptors synapse in both rostral and caudal brainstem regions (Luo et al. 1995) while 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,II and III does not readily fit into a rostral innocuous/caudal nociceptive paradigm but rather that both innocuous and noxious muscle afferent feedback is distributed along the entire dorsomedial portion of the brainstem.

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Figures

Fig. 1. Schematic diagram of the experimental methodology used for in vivo intraaxonal recording and staining in an anesthetized rat. A. A sharp microelectrode (a) was used to impale the central axonal process (b) of a single masseter muscle primary afferent neuron in the brainstem. 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 (grey area). C. response of the muscle afferent axon to mechanical stimulation (1765mN) within the muscle receptive field. D. four superimposed sweeps showing electrical activation of this axon at threshold stimulation of the masseter nerve (62T). Asterisk denotes stimulus artifact, arrow points to two failures of masseter nerve stimulation to evoke an action potential. Membrane potential for this axon was -47mV, conduction velocity was 26M/s.

Fig. 2 Reconstruction of a IIIHTM_{Vp-Vc} trigeminal ganglion muscle afferent neuron in sagittal view using camera lucida. This neuron enters the brainstem 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 = 1mm

Fig. 3 Neuronal reconstructions of representative group III masseter muscle mechano-nociceptors using image analysis software. These axons were activated by high-threshold stimulation of the masseter nerve and noxious mechanical stimulation of the masseter muscle. A. Single intraaxonally labeled HTMIIIIVo-Vc type axon activated by masseter nerve stimulation at 94T with a conduction velocity (CV) of 24 M/s. Darkened line indicates the parent axon and terminal collaterals are diagrammatically indicated. Axon collaterals project to dorsomedial portions of caudal Vo as well as Vi and Vc. B. Single labeled HTMIII axon with a CV of 23 M/s exhibiting a slight variant of the axon morphology shown in A. This axon was activated at 77T and terminates only in Vi and Vc. C. Single HTMIIIVp-Vc type axon (CV=24 M/s) which was activated by nerve stimulation at 63T and projects to the dorsomedial Vp, Vo, Vi and Vc. D. HTMIIIVp-Vc axon activated at 60T with a CV of 29 M/s. Amb - nucleus ambiguus, IO - inferior olive, Vmo - trigeminal motor nucleus, Vtr - spinal trigeminal tract, XII - hypoglossal nucleus.

Fig. 4 Intracellularly stained axon collaterals of masseter muscle HTM neurons. A. axon collateral (red arrow) of a IIIHTMIVp-Vc masseter muscle afferent with en passant and terminal boutons (purple arrows) located in the dorsomedial Vp. Note the dense, restricted distribution of boutons. B. compressed projection of a confocal image stack showing an intracellularly-stained IIIHTM muscle afferent axon collateral (red) in Vo in association with Nissl-stained neurons.
(green). C. photomontage derived from five images of a IIIHTM masseter muscle afferent. This axon collateral (red arrow) with boutons (some indicated by purple arrows) was located in Vi. D. compressed confocal image stack of IIIHTM masseter muscle mechanoreceptor axon collateral (red) amongst Nissl-stained neurons in lamina V of Vc. Insets in A-D show the location of axon (red). Green outline denotes the Vtr, Blue outlines indicate: A:Vmo, B:VII, C:Aud, D:XII  Scale bars: A: 100µm, B,D: 20µm, C: 10µm.

Fig. 5 Diagrammatic representation of masseter muscle high-threshold mechanoreceptor afferent types. A. IIIHTM_{Vp-Vc} afferent type has axon collaterals distributed throughout the brainstem trigeminal sensory nuclear complex. B. IIIHTM_{Vo-Vc} afferent type has axon collaterals only in the caudal brainstem. Vertical lines emerging from the main axon indicate regions where axon collaterals emerge, not single axon collaterals. Horizontal bars show the rostro-caudal distribution of axon collaterals.

Fig. 6 Combined intracellular and synaptophysin labeling. A. masseter muscle afferent axon collaterals intracellularly labeled with biotinamide (red) and synaptophysin immunolabeling (green). Region devoid of synaptophysin labeling (arrow) is the location of a neuronal soma., B-F. Sequence of optical sections (0.5µm spacing) through the boxed region in 6A. Arrows point to a synaptic vesicle cluster, scale bar = 10µm and applies to all images.

Fig. 7 Colocalization indices derived from synaptic vesicle clusters within intracellularly stained axon terminals. Note that colocalization indices are substantially higher than random for muscle
spindle afferent boutons in the trigeminal motor nucleus (Vmo) and in all regions for both
IIIHTM_Vp-Vc and IIIHTM_Vo-Vc masseter muscle afferents. Asterisks indicate colocalization indices
which are statistically different from indices obtained from randomly shuffled images.
Table 1 - Electrophysiological Properties of High Threshold Masseter Muscle Afferent Neurons

<table>
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<th>labeled population</th>
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<tr>
<td></td>
<td>n</td>
<td>mean</td>
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<td>threshold for masseter nerve stimulation (T)</td>
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Figure 1
Figure 3
Figure 4
Figure 5
Figure 7