Increase in membrane potential oscillations and spontaneous firing of muscle spindle afferents after injections of acidic saline into the masseter muscle

Somayeh Sadeghi
Department of Dentistry, McGill University, Montreal
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1.0 ABSTRACT

Cell bodies of Group I and II afferents of muscle spindles of jaw muscles are located in the mesencephalic trigeminal nucleus (NVmes). Their intrinsic electrical properties include resonant properties that produce high frequency oscillations which sometimes lead to rhythmic bursting. Increases in these behaviours in spinal dorsal root ganglia sensory afferent neurons have been linked to the appearance of neuropathic pain. We postulated that similar changes may occur in muscle spindle afferents in a model of non-neuropathic chronic muscle pain, and so investigated the effects of algesic injections into the masseter muscle on the intrinsic properties of NVmes afferents. We adapted the method of dual intramuscular acidic saline injections (pH 4, 2-3 days apart) developed by Sluka et al. to produce long-lasting mechanical hypersensitivity of rat masseter muscles. 

In vitro whole-cell current clamp recordings were used to study the basic electrophysiological properties of somata of jaw closing muscle spindle afferents. In Experiment 1, NVmes cells were recorded from animals that were sacrificed from between < one day to 35 days after the second injection. While some basic firing characteristics were similar between groups, differences were noted. Neurons recorded from the acidic saline group (n = 167 neurons) had more hyperpolarized resting membrane potentials than those of control neurons (n = 198). Threshold firing for bursting and high frequency membrane oscillation, inward and outward rectification were shifted to more hyperpolarized values. At similar membrane potentials, the amplitude of the high frequency membrane
oscillations were significantly greater and spontaneous firing occurred occasionally (10%) in the acidic group, but never in the controls. Electrophysiological changes appeared within the first day after the first injection and lasted up to 5 weeks. Experiment 2 was carried out on rats that had received a single injection of acidic (n = 17) or of normal (n = 14) saline. One to two days after injection, significant changes had occurred in membrane properties of the experimental group, showing that the spindle afferents reacted to a single exposure to acidic saline. The results support the hypothesis that increases in membrane oscillations and associated spontaneous firing may be associated with the development of chronic muscle pain.

1.0 ABRÉGÉ

Les corps cellulaires des afférences fusoriales des groupes I et II des muscles de la mastication sont situés dans le noyau trigéminal mésencéphalique (NVmes). Ces afférences ont des propriétés de résonnance qui leur permettent de produire des oscillations à haute fréquence qui peuvent parfois mener à une décharge en bouffées rythmiques. L'apparition de la douleur neuropathique a été associée à l'augmentation de l'incidence de ce type de comportement dans les neurones sensoriels afférents du ganglion spinal radiculaire. Notre hypothèse postule que des changements similaires peuvent se produire dans les afférences des fuseaux neuromusculaires dans un modèle de douleur musculaire chronique,
non-neuropathique. Nous avons donc examiné les effets produits par des injections algésiques dans les muscles des masséters sur les propriétés intrinsèques des afférences du NVmes. Nous avons adaptés la méthode de l'injection intramusculaire bilatérale d'acide salin (pH 4, 2-3 jours d'intervalle) développée par Sluka et al. pour produire une hypersensibilité mécanique de longue durée dans les muscles masséters. Des enregistrements de cellule entière In Vitro en courant impose, ont été utilisés pour étudier les propriétés électro physiologiques de base des corps cellulaires des afférences neuromusculaires des muscles de la mastication. En premier lieu, (expérience 1), les cellules du NVmes ont été enregistrées chez des animaux, de 1 à 35 jours après la deuxième injection d'acide salin. Alors que la plupart des propriétés de base sont restées stables, quelques différences ont pu être observées. Les neurones du groupe expérimental avec injections d'acide salin (n=167) ont présnetés des potentiels de repos membranaires plus hyperpolarisés que les neurones des groupes de contrôle (n=198). Les seuils de décharge, de bouffées, et de déclenchement des oscillations membranaires, ainsi que les potentiels de rectification entrante et sortante, sont tous passés à des valeurs plus hyperpolarisées qu'auparavent. Dans le cas de potentiel membranaire semblable, l'amplitude des oscillations membranaires à haute fréquence a été significativement plus élevée dans le groupe expérimental, et une décharge spontanée au potentiel de repos a été observé chez 10% des neurones de ce groupe, comparativement aux groupes de contrôle où aucune n'a été observée. Les changements électrophysiologiques apparaissent entre le premier jour après la
première injection d'acide salin, et peuvent être observés jusqu'à 5 semaines suivant l'injection. Dans la seconde partie (expérience 2), les rats n'ont reçu qu'une seule injection bilatérale d'acide salin (n=17) ou de salin neutre (n=14). Une à deux journées suivant l'injection de salin, des changements significatifs dans les propriétés membranaires sont apparus chez le groupe expérimental, démontrant que les afférences fusoriales neuromusculaires ont réagi à une seule injection du traitement d'acide salin. Ces résultats soutiennent l'hypothèse que l'augmentation des oscillations membranaires et une activité spontanée du neurone NVmes connexe, pourrait être associée au développement de la douleur chronique musculaire.
2.0 INTRODUCTION

Perception allows mankind and other animals to experience and recognize their surroundings. Each of the five senses plays a great role in the perception of life, but they work together to deliver our perception of the external world. Personal experience and learning effects how we view a situation. The activation of a receptor cell is the first step to perception (Ragsdale 2005) but the sensory information is then processed at several levels in the Central Nervous System (CNS); beginning in the primary afferent neurons (Ragsdale 2005). However, how one perceives the world is not necessarily a good indication of reality. "Sensations are set by the encoding functions of sensory nerve endings, and by the integrated neural mechanics of the central nervous system. Afferent nerve fibers are not high-fidelity recorders, for they accentuate certain stimulus features, neglect others. The central neuron is a story-teller with regard to the nerve fibers and it is never completely trustworthy, allowing distortions of quality and measure. Sensation is an abstraction, not a replication, of the real world" (Mountcastle 1975).

2.1 Cranial Nerves

The following description is based on Kandel et al. (2000). The spinal nerves end at the second cervical vertebra, hence the somatic and visceral sensory
and motor innervations of the head is supplied by the cranial nerves. All cranial nerves, except for the trochlear nerve (IV) which leaves the midbrain from its dorsal surface, exit the brainstem at a specific location in numerical order from the ventral surface. There are many similarities between the motor/sensory components of the brainstem and the spinal cord but there are also some significant differences between the two. A sensory ganglion is associated to each cranial nerve with a sensory function that is located along the length of each nerve or immediately before the entrance into the skull; they are similar to Dorsal Root Ganglia (DRG) of the spinal nerves. The main cranial nerves with somatosensory functions are the trigeminal nerve (V), the glossopharyngeal nerve (IX), and the vagus nerve (X).

### 2.1.1 The Vth Cranial Nerve

The fifth (V) cranial nerve, also known as the Trigeminal Nerve is the largest of the cranial nerves. It contains both sensory and motor axons, and leaves the brainstem in two roots, one being the motor root and the other the sensory root. It is called the “Trigeminal” because it has three main branches, the Ophthalmic, Maxillary, and Mandibular nerves. Each of these innervates a well-defined territory on the face and the head, including teeth and the mucosa of the oral and nasal cavities. The brainstem trigeminal sensory nuclei extend through the whole of the brainstem, from the midbrain to the medulla and down into the
rostral spinal cord. These are divided into three parts; from caudal to rostral they are the spinal trigeminal nucleus, the principal sensory trigeminal nucleus, and the trigeminal mesencephalic nucleus. The muscles of mastication (the masseter, temporalis, medial and lateral pterygoids, anterior digastrics and mylohyoid muscles) and some muscles of the palate (tensor Veli palatine), inner ear (tensor tympani), are all innervated by trigeminal motor nucleus and its motor root (Kendel et al. 2000).

2.2 The Trigeminal Mesencephalic Nucleus

The mesencephalic (MesV) trigeminal nucleus is made up of a thin column of cell bodies of primary afferent neurons in the midbrain that runs along the lateral surface of the periaqueductal gray matter, but which then becomes more compact as it approaches the V\textsuperscript{th} motor nucleus in the pons. They are the only primary sensory afferent neurons to have their cell bodies located within the CNS (Kandel et al. 2000).


A number of authors (Byers et al. 1986, Nomura and Mizuno 1985, Szentágothai 1948 Appenteng et al. 1985, Appenteng et al. 1978, Cody et al. 1972, Corbin 1940, Jerge 1963, Kidokoro 1981, Nakamura et al. 1967, Shigenaga et al. 1989, Yoshida et al. 1987) have shown that the cell bodies of the MesV trigeminal nucleus are large unipolar cells that lack dendritic processes in adult animals. Their stem axons divide into a peripheral process and several central processes, at irregular distance from the soma (200-300μm) (Baker and Llinas 1979). Both spindle afferents and periodontal afferents have large myelinated axons. The central branches of the axons from some spindle afferents (Jerge 1963) make synaptic contact with jaw-closing motoneurons (Bae et al. 1996) in the trigeminal motor nucleus, providing monosynaptic sensory feedback. Stimulation of muscle spindles by stretching the muscle can initiate a stretch reflex, the Jaw Jerk or Jaw

The studies of Corbin (Corbin 1940) and Corbin and Harrison (Corbin and Harrison 1940) revealed a great deal about the function of the MesV nucleus. They showed that the MesV root fibers pass into the muscle branches of the fifth nerve, and also into the sensory branches that supply the teeth, gums and hard palate (superior alveolar, inferior alveolar and palatine nerves). They demonstrated that MesV sensory neurons are activated by jaw-opening movements, or by applying pressure in the mouth. They suggested the association of the MesV nucleus with the jaw reflex by showing that lesions of MesV abolishes the reflex (Harrison and Corbin 1942). More recent studies have demonstrated the ability of MesV neurons to function as interneurons in networks responsible for the orofacial musculature (Kolta et al. 1995, 2000).

2.3 Gap Junctions and Synapses

It has been reported that there are somato-somatic and somato-axonic contacts between cells of MesV (Weinberg 1928, Kappers et al. 1936, Crosby et al. 1962, Woodburne 1936). In some cases the complexes were shown to be extensive and produced relatively large clusters of neurons (Capra et al. 1985).
Many of the MesV cells are in very close proximity of each other (50μm), forming clusters between two or more neighbouring cells (Baker and Llinas 1979, Weinberg 1928, Hinrichsen and Larramendi 1969, Woodburne 1936, Torvik 1956, Hinrichsen and Larramendi 1968). Furthermore, these contacts have the appearance of gap junctions when viewed under the electron microscope (Hinrichsen and Larramendi 1968, Hinrichsen and Larramendi 1969, Liem et al. 1991), which would allow action potentials to pass from one neuron to another (Baker and Llinas 1979, Hinrichsen and Larramendi 1969). Another feature of the MesV cells that distinguishes them from the cell bodies of primary afferents located in the DRG (Cajal 1909, Torvik 1956, Yamamoto and Kondo 1989) is that they also receive chemical synapses (Torvik 1956, Hindrichsen and Larramendi 1968). Bennett (1972) suggested that because synapses are found next to gap junctions, they may regulate the degree of electrical coupling. Furthermore, there is evidence that chemically-mediated cross-excitation also takes place between these neurons (Puil and Spiegelman 1988).

response to the synaptic actions independent of the activity of their peripheral receptors (Pelkey et al. 1998). This was finally shown by Verdier et al. (2003) in an in vitro slice preparation. They found that synaptic inputs to muscle primary afferent neurons in MesV from neighbouring areas are mainly excitatory and that they can cause firing.

2.4 Intrinsic Electrical Properties

Morphologically there is a resemblance between the cells from the MesV nucleus and DRG cells, with which they are homologous (May and Horsley 1910, Allen 1919, Clark 1926, Schneider 1928, Sheinin 1930, Weinberg 1928). The similarities extend to the intrinsic electrical properties of the large cells of MesV and the DRG. Both type of cells show inward rectification on membrane hyperpolarization (Khakh and Henderson 1998, Tanaka et al. 2003, Verdier et al. 2004). They also have resonant properties (Hutcheon and Yarom 2000) that produce high frequency subthreshold membrane oscillations (Verdier et al. 2004). These sinusoidal fluctuations of membrane potential sometimes lead to rhythmic bursting or repetitive discharge at slightly depolarized potentials (Wu et al. 2001, Amir and Devor 1997, Puil and Spieglman 1988, Puil et al. 1989, Wall 1983, Pedroarena et al. 1999, Verdier et al. 2004). The depolarizing phase of the oscillation in both populations depends on a persistent sodium current (I_{NaP}), whereas re-polarization is potassium dependent (Pedroarena et al. 1999).
properties are voltage-dependent and can be regulated by synaptic inputs (see above).

2.5 Pain

2.5.1 Acute and Chronic Pain

Pain is often divided into two categories, Acute Pain and Chronic Pain. Acute pain is a short term pain, usually with an easily identifiable cause, that acts as a warning of on-going tissue damage. It is a symptom of a disease process and is usually well localized. It occurs secondary to chemical, mechanical and thermal stimulation of nociceptors in the terminals of small diameter thinly myelinated (Aδ) or unmyelinated C fibers (Ragsdale 2005, Purves et al. 2001). In contrast, chronic pain persists long after the original injury has healed, or it may have no identifiable cause. Chronic pain is usually classified as neuropathic or non-neuropathic. Neuropathic pain is a sensation evoked by damage or pathological changes in peripheral nerves or in the CNS. Among the common symptoms is spontaneous pain, which is often described as a burning or tingling sensation, and a hypersensitivity to touch or cold, (allodynia) (Purves et al. 2001). The most striking and mystifying example of neuropathic pain is perhaps the "phantom limb pain syndrome." This occurs when an arm or a leg is amputated;
however the brain still perceives pain coming from the missing limb (Purves et al. 2001).

2.5.2 Allodynia and Hyperalgesia

Allodynia is the pain sensation that is evoked by a stimulus that is normally below the pain threshold, while hyperalgesia is an increase in the sensitization evoked by a normally painful stimulus. If allodynia and hyperalgesia are induced from a site of injury, they are referred to as primary allodynia and hyperalgesia (Cervero et al. 2003). Secondary allodynia and hyperalgesia are evoked from a larger area neighbouring or distant from the injury site (Cervero et al. 2003).

There is evidence that pain signalling is not exclusively transmitted by nociceptors. Although this may be the case under normal conditions, there is good evidence that large myelinated fibers contribute to the signalling of pain following nerve injury. Campbell et al reported that hyperalgesia could be eliminated by a selective blockade of Aβ fibers, but not by a selective blockade of the C and Aδ fibers. Furthermore, the short latency of the pain response also pointed transmission by Aβ fibers (Campbell et al. 1988).
There are reports of changes occurring in the dorsal horn after nerve injury (Cook et al. 1987, Wall and Devor 1983, Woolf 1983, Cervero and Laird 1996b, Treede et al. 1992). These may strengthen the synaptic link between low-threshold mechanoreceptors and the central pain signalling pathways; though this possible reorganization in the CNS does not account for the fact that hyperalgesia has the potential to emerge within seconds after injury (Cervero et al. 2003, Ochoa et al. 1996). The characteristic of these changes suggests that incoming activity from low-threshold mechanoreceptor afferents from an area of secondary hyperalgesia gains access to the nociceptive sensory channel (Campbell et al. 1988, Cervero et al. 2003). However, a key component of developing and sustaining secondary hyperalgesia is incoming nociceptive activity from an area of primary hyperalgesia (Dubner and Ruda 1992).

2.5.3 Animal Models of Neuropathic Pain

There are two important animal models of neuropathic pain: the chronic constriction injury model where a loose ligature is placed around the full sciatic nerve (Xie et al. 1995, Study and Kral 1996, Kajander and Bennett 1992, Kajander et al. 1992, Petersen et al. 1996, Zhang et al. 1997) and the spinal nerve lesion model (Chung model of pain) which was developed by tightly ligating one (L5) or two (L5 and L6) segmental spinal roots (Lee et al. 1999, Liu et al. 2000a). Both cause long-lasting behavioural signs of mechanical allodynia, heat
hyperalgesia, cold allodynia, and ongoing pain. It has been suggested that neuropathic pain is causal by ectopic action potentials initiated at the site of the nerve lesion (Devor and Seltzer 1999, Jänig et al. 1996), or elsewhere along the axon, or from altered processing in the DRG (Gracely et al. 1992). Somata of primary sensory neurons in DRGs seldom fire spontaneously (LaMotte and Campbell 1978); however, following peripheral nerve injury, the prevalence of intrinsic oscillations increases, resulting in spontaneous firing and repetitive discharges. These can occur in both the cell bodies of injured A fibers and intact C fibers (Liu et al. 2000). Spontaneous ectopic discharge following nerve injury has been noted in skeletal muscle afferents as well as in skin afferents (Michaelis et al. 1995, Amir et al 1999). It has been reported that DRG neurons that failed to show oscillations, even when deeply depolarized, were unable to generate continuous discharge, signifying the importance of the oscillatory process for repetitive firing in this type of neuron. Ectopic firing has been directly linked to the development of allodynia and hyperalgesia (Devor and Seltzer 1999, Sheen and Chung 1993, Sukhotinsky et al. 2004, Nordin et al. 1984, Kuslich et al. 1991, Devor 1994, Xie et al. 1995, Study and Kral 1996), and it has been reported that prevention of ectopic firing eliminates both allodynia and hyperalgesia (Sheen and Chung 1993, Gracely et al. 1992). Gracely et al performed experiments on 4 patients diagnosed with reflex sympathetic dystrophy where they made sensory assessments before and during diagnostic tourniquet-cuff and local anaesthetic block. Their observations suggested that continuous input from a painful focal point is responsible for both spontaneous pain and maintenance of altered central
processing that yields mechno-alldynia, other sensory abnormalities and disorders of motor function. They concluded that blocking the peripheral input causes the central processing to regress to normal and eliminates the symptoms for the duration of the block (Gracely et al. 1992).

2.5.4 Muscle Spindle Afferents and Chronic Muscle Pain

As described in section 2b, MesV muscle spindle afferent cell bodies show the same high frequency oscillations and capacity for ectopic firing as large diameter dorsal root ganglion cells. Therefore, we speculated that they could play a role in chronic muscle pain. There is preliminary evidence that muscle spindles contribute to one form of persistent pain, Delayed Onset Muscle Soreness (DOMS), which often occurs after heavy exercise and which usually resolves within several days (Jones et al. 1997, Proske 2005, Weerakkody et al. 2001). Heavy exercise can damage sacromeres as well as the cell membrane of muscle fibers (Weerakkody et al. 2001), causing a local inflammatory reaction in the region (Smith 1991), including invasion by macrophages and monocytes (Stauber et al. 1988). Consequently, there is a tissue breakdown that could sensitize the nociceptors by the release of chemicals such as prostaglandins in the injured area (Stauber et al. 1988, Mense 1996). Weerakkody et al (2001) performed experiments on DOMS in the gastrocnemius muscle using vibration to stimulate muscle spindle afferents. They measured the pain threshold before, during, and
after applying pressure the sciatic nerve to block large diameter afferents. They noted that in an unexercised muscle, vibration reduced pain caused by hypertonic saline injections, but that it increased it in DOMS. They also showed that the pressure-pain threshold was significantly increased during the large-diameter afferent block in DOMS (Barlas et al. 2000). A recent study by Semmler et al. (2007) also suggested altered motor unit activation after eccentric exercise. Their findings showed that eccentric exercise impaired motor control and altered neural drive to elbow flexor muscles.

2.5.5 An Animal Model of Chronic Muscle Pain

Intramuscular injection of hypertonic saline was first introduced by Kellgren (1938) to model deep tissue pain in humans and this model is now widely used to mimic myofascial pain (Stohler et al. 1992, Kellgren 1938, Graven-Nielsen et al. 1997a, Graven-Nielsen et al. 1997b). Furthermore, intramuscular injections or infusion of hypertonic saline has been used to induce pain in the masseter muscle (Stohler et al. 1992, Svensson et al. 1998). Patterns of radiation and pain reports are similar to those found in TMD patients, and masticatory patterns are changed in a manner similar to those observed in chronic TMD patients (Svensson et al. 1998, Lund et al. 1991). Similar changes in mastication are evoked in decerebrate rabbit by saline injections into the masseter muscle. Westberg et al. (1997) showed that the burst characteristics of trigeminal
motoneurons and interneurons during fictive mastication are modified in parallel. Lund et al. (1991) explained the changes in motor activity by proposing that the interaction between nociceptive afferents and the masticatory central pattern generator network controls the pre-motor neurons to agonist and antagonist motoneurons in a reciprocal way.

The experimental model we used to induce chronic muscle pain was a modification of the Kellgren developed recently by Sluka et al. (2001). They showed that two injections of acidic saline into one gastrocnemius muscle of rats 2 to 5 days apart produces a long-lasting bilateral hyperalgesia (Sluka et al. 2001). To measure the level of hyperalgesia, they examined paw withdrawal latency to radiant heat (heat hyperalgesia) and withdrawal threshold to mechanical stimuli (mechanical hyperalgesia). They used the model to show that the bilateral mechanical hyperalgesia of the paw is sensitive to spinally administered NMDA and non-NMDA ionotropic glutamate receptor antagonists (Sluka et al. 2001, Skyba et al. 2002).
3.0 MATERIALS AND METHODS

3.1 Labeling of cell:

Somata of jaw closing muscle spindle afferents in the mesencephalic trigeminal nucleus (NVmes) were retrogradely labeled by injecting a tracer (Chlora toxin conjugated to Alexa Fluor 488 or DilC18(3)) in the masseter muscles of 1-3 days old Sprague-Dawley rats.

3.2 Brainstem Slice Preparations:

Animals were decapitated and the brain was quickly removed. Coronal brainstem slices (250-350µm) were prepared with a vibratome (Leica VT100S). The brain was sectioned in ice cold (4°C), modified sucrose-based artificial cerebrospinal fluid (ACSF- in mM; 225 sucrose, 3KCl, 1.25 KH2PO4, 4MgSO4, 26 NaHCO3, and 25 D-glucose). Slices were transferred at room temperature to a submerged type chamber for whole cell patch-clamp recordings and continuously perfused with oxygenated (mixture of 95% O2 and 5% CO2) normal ACSF (in mM: 126 NaCl, 3KCl, 1.25 KH2PO4, 1.3MgSO4, 2.4 CaCl2, 26NaHCO3, and 25 D-glucose).
3.3 Protocol:

Experiment 1: Effect of Acidic Saline Injection on Masseter Muscle Spindle Membrane Properties: Long-Term Changes.

A total of 83 new-born rats were used, and data was gathered from 72. Following the injections of a tracer 1-3 days after birth, the animals were divided into 4 groups. Group A received no injections, while group B got injection of buffered normal saline (10μl, 0.9% NaCl at pH 7.2) into both left and right masseter muscle. Animals of group C animals received an injection of 10μl of saline at neutral pH into the masseter muscle on one side, and 10μl acidic saline (pH 4) on the opposite side. Half received the acidic saline on the left and the neutral pH saline on the right; the other half received the acidic saline on the right and the neutral pH on the left. Finally, acidic saline with a pH of 4 was injected into both left and right masseter muscle of the animals of group D (Table 1). Following the protocol established by Sluka et al., animals receiving injections got their first dose the day after the labeling of the cells followed by a second dose two days later. Electrophysiological experiments were carried out at 7 different times after the second injection: T1: 2-8 hours; T2: 24 hours; T3: 2-5 days; T4: 6-8 days; T5: 9-11 days; T6: 12-14; T7: 32-35 days after. The person carrying out the recordings and data analysis was not blind to treatment. Refer to Table 1.
Table 1. Experiment 1: Animal groups and neuronal populations

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>A (n=9)</th>
<th>B (n=15)</th>
<th>C (n=33)</th>
<th>D (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Non-injected saline</td>
<td>Normal saline</td>
<td>Normal saline</td>
<td>Acidic saline</td>
</tr>
<tr>
<td>Side</td>
<td>Bilateral</td>
<td>Bilateral</td>
<td>Unilateral</td>
<td>Unilateral</td>
</tr>
<tr>
<td>Initial neuronal population</td>
<td>1 (n=57)</td>
<td>2 (n=54)</td>
<td>3 (n=87)</td>
<td>4 (n=100)</td>
</tr>
</tbody>
</table>

Four animal groups (A-D) received different treatments. The number of animals in each group is indicated between brackets. Cells recorded from animals from each of these groups formed a distinct neuronal population. Two populations came from group C, because a different treatment was given on each side. The number of cells in each population is indicated between brackets.


After the preliminary analyses of data from Experiment 1 showed significant differences between neutral and acid saline neurons immediately after the second saline injection, a second experiment was performed in which acid saline treatment was given only once. Six animals were prepared as for Group B (bilateral neutral saline) and D (bilateral acid saline) above. Recordings were made one and two days after the first and only injection (T-1 and T2,
respectively). The person carrying out the recordings and data analysis was blind to treatment.

3.4 Electrophysiology:

Labeled somata were visualized using a fixed stage microscope (Eclipse E600FN, Nikon) coupled with a 40X water immersion lens. Labeled cells were identified using epifluorescence and approached under infra-red microscopy for recordings. The image was enhanced with an infrared-sensitive CCD camera and displayed on a video monitor. Whole cells patch-clamp recordings in current-clamp mode were made using an AxoClamp-2B amplifier (Axon Instruments, Foster City, CA). Patch electrodes (3.5MΩ-8.5MΩ) were pulled from borosilicate glass capillaries (1.00mm OD, 1.12mm ID; World Precision Instruments, Sarasota, FL) on a Sutter P-97 puller (Sutter Instruments Company, Novato, CA) and filled with a K+-gluconate based solution (in mM: 140K+-gluconate, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 2 ATP [di(tris) salt hydrate], 0.4 GTP (tris salt)). All chemicals were supplied by SIGMA. The voltage drop induced by a current pulse (-100pA, 200ms) was used to measure the resistance of the electrode in the Bridge Mode of an AxoClamp-2B amplifier (Axon Instruments, CA). A small positive pressure was applied to the back of the pipette in order to preclude any material from attaching to the pipette while it was directed towards the cell. Once the electrode tip reached the surface of the tissue, suction was gently applied until a high resistance of (1-5GΩ) seal was made, then suction was applied to break into
the cell body. Incrementing and decrementing depolarizing and hyperpolarizing currents steps and ramps were injected in every cell to measure basic electrophysiological properties.

3.5 Data Acquisition and Analysis

Whole-cell current and voltage recordings were attained through a Digidata 1322A interface and analyzed with Clampex 9 software (Axon Instruments). Only cells with a resting membrane potential (RMP) below -50mV were used in the experiments. The input resistance is measured by the slope of the linear portion of the I/V relationship (Fig 1B) obtained by plotting membrane voltage changes produced by steps of current (0.1nA, 1s; Fig 1A), as well as, by measuring the slope of the voltage changes produced by ramp current injections at three different points as indicated in figure 1C. Inward and outward rectifications were considered to occur at inflexion points where slopes changed in the RAMP I-V relationship (Fig 1B; Fig 1C). Threshold was considered as the membrane potential at which spiking was produced by injection of incrementing depolarizing pulses (steps of 0.1nA) into the patched cell. In order to examine the basic firing characteristics of the cells, single spikes were evoked by brief intracellular pulses (1ms) at holding potential. The following measurements were made: firing threshold, peak amplitude of the action potential from resting membrane potential (RMP), action potential half-width, amplitude of the after-hyperpolarization
(AHP; measured from RMP), and AHP duration (measured as the time to RMP (Fig 1D).

Incrementing 1 s current steps were also used to measure the threshold of bursting when present and of the high-frequency membrane oscillations. Maximum amplitude and frequency of the oscillations (Fig 1E) were measured on the screen at the membrane potential at which the amplitude appeared to be maximal. Means were calculated from three cycles with greatest amplitude (see inset). Patterns of spontaneous firing and firing during maintained depolarization were described.
Figure 1

A. PULSE I-V

B. Current-voltage relationship

C. RAMP I-V

D. Waveform

E. Oscilloscope
3.6 Statistical Analysis

Parametric data was expressed as mean ± Standard Error (SE). Normality of distribution was tested for all variables in all groups. When parametric tests could be used, between groups comparisons were performed with ANOVAs or repeated measured ANOVAs. For pair wise comparison, independent t-tests or Mann-Whitney U tests were used. Chi-squared tests were used to compare patterns of firing between treatment groups. Probabilities of alpha-type errors of <0.05 were considered to be significant. All analyses were carried out by a person naïve to the aims of the experiment.
3.7 OBJECTIVE

The general hypothesis that we have begun to test is that changes in the phenotype of muscle spindle afferents could be an essential factor in the genesis of chronic muscle pain. The principle objective of the current study was to see if the induction of chronic muscle pain was accompanied by an increase in the amplitude of high frequency oscillations and spontaneous firing in MesV muscle spindle afferent neurons. A second objective was to describe changes in neuronal membrane properties and the time course of these changes.

The study was carried out on newborn rats. First, injections of tracer were made into both masseter muscles in order to label the cell bodies of muscle spindle afferents and motoneurons by axonal transport. Some of these animals were used as un-operated controls; the others were divided into an active control group that received injections of normal saline into the masseter, and a pain group that were injected with acidic saline. Later, the animals were decapitated, and slices of the brainstem were prepared for in vitro recording from MesV with patch clamp electrodes.
4.0 RESULTS

4.1 Experiment 1

A total of 365 neurons were used in the analyses. They were first separated into seven populations depending on the groups of animals from which they came, and in the case of neurons from Group C, the side on which the acid or normal saline was injected (Table 1).

4.1.1 Assessment of Pain:

Several observations indicated that the injection of acidic saline produced chronic pain. Animals injected with this algesic substance tended to rub their jaw more often and to have very minimal movements. They experienced sudden shakes and shivering which were not observed in the control group under similar conditions. Exploratory behaviors in new environments were also reduced in these animals.

4.1.2 Initial Between-Group Comparisons of Neuronal Properties

We first compared the data from 12 measurements made on initial neuronal subgroups within the sham control and experimental populations (Table
1) using two-way ANOVA for Group and Time. Group alone had no significant effect on any of the variables between initial groups 2 (bilateral normal saline) and 3 (unilateral normal saline), so they were combined into one sham control group to simplify further analyses. In comparisons between groups 4 (unilateral acidic saline) and 5 (bilateral acidic saline), there was a small but significant (P=0.0065) difference in spike threshold between groups (mean ± SE= -53.2±5.5 for the unilateral injections vs. -54.9±4.5, for the bilateral injections). However, this was the only significant effect out of 24 statistical tests, so we decided to combine groups 4 and 5 into one experimental group.

We carried out univariant analyses to compare non-injected controls, sham controls and experimental animals. Table 2 shows that there were significant differences between the experimental and the two control groups in seven parameters. The mean RMP of the experimental group was about 8mV more hyperpolarized than both controls, and there was a significant hyperpolarizing shift of a similar magnitude in the firing threshold. Inward and outward rectification also occurred at membrane potentials that were considerably more hyperpolarized in the experimental group. The significant differences were also seen in the thresholds for membrane oscillations and the associated burst firing; both occurred at lowest membrane potentials in the experimental group. The maximum oscillation amplitude was also significantly greater in the experimental group. Note that the probabilities of α-type error were extremely low. Figure 3A
shows the amplitude of oscillations obtained at similar membrane potentials in one sham control and one experimental neuron.

There were no significant differences in input resistance, or in the properties of the action potential and the following after-hyperpolarization between the Sham and Experimental Groups; however, significant differences between the Experimental and Non-injected Controls were found for Action Potential Half-width, After-Hyper-polarization Amplitude and Duration. There were also significant differences in 5 variables between the two control groups. The reason for this became apparent in the next analysis.

Table 2. Experiment 1: Neuronal properties.

<table>
<thead>
<tr>
<th>VARIABLES/ GROUPS</th>
<th>Non-injected Control (n = 57)</th>
<th>Sham Control (n = 141)</th>
<th>Experimental (n = 167)</th>
<th>P Non-injected vs Sham</th>
<th>P Non-injected vs Experimental</th>
<th>P Sham vs Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Membrane Potential, mV</td>
<td>-56.16 ± 0.45</td>
<td>-55.80 ± 0.27</td>
<td>-63.30 ± 0.31</td>
<td>0.50</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Firing Threshold, mV</td>
<td>-45.56 ± 0.68</td>
<td>-44.64 ± 0.42</td>
<td>-53.89 ± 0.40</td>
<td>0.25</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Input Resistance, MΩ</td>
<td>86.30 ± 5.94</td>
<td>77.28 ± 2.90</td>
<td>81.62 ± 3.12</td>
<td>0.18</td>
<td>0.49</td>
<td>0.31</td>
</tr>
<tr>
<td>Action Potential Amplitude, mV</td>
<td>78.81 ± 1.39</td>
<td>83.05 ± 1.02</td>
<td>81.50 ± 0.97</td>
<td>0.015</td>
<td>0.11</td>
<td>0.27</td>
</tr>
<tr>
<td>Action Potential Half-Width, ms</td>
<td>0.95 ± 0.04</td>
<td>0.79 ± 0.02</td>
<td>0.81 ± 0.02</td>
<td>0.001</td>
<td>0.006</td>
<td>0.35</td>
</tr>
<tr>
<td>After Hyper-Polarization Duration, ms</td>
<td>75.88 ± 6.12</td>
<td>47.85 ± 2.63</td>
<td>43.75 ± 2.38</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.25</td>
</tr>
<tr>
<td>After Hyper-Polarization Amplitude, mV</td>
<td>8.60 ± 0.36</td>
<td>10.32 ± 0.28</td>
<td>10.34 ± 0.22</td>
<td>0.0003</td>
<td>0.0001</td>
<td>0.95</td>
</tr>
<tr>
<td>Inward Rectification Threshold, mV</td>
<td>-71.11 ± 0.76</td>
<td>-69.51 ± 0.55</td>
<td>-77.27 ± 0.57</td>
<td>0.091</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Outward Rectification Threshold, mV</td>
<td>-48.71 ± 0.86</td>
<td>-48.15 ± 0.68</td>
<td>-56.09 ± 0.62</td>
<td>0.61</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Oscillation Threshold, mV</td>
<td>-56.11 ± 0.66</td>
<td>-56.25 ± 0.35</td>
<td>-63.86 ± 0.38</td>
<td>0.85</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maximum Amplitude of Oscillation, mV</td>
<td>0.40 ± 0.03</td>
<td>0.66 ± 0.03</td>
<td>1.49 ± 0.07</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bursting Threshold, mV</td>
<td>-48.74 ± 1.14</td>
<td>-46.48 ± 1.20</td>
<td>-57.25 ± 0.69</td>
<td>0.18</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Mean ± SE of measurements made on all cells from the three neuronal populations were compared using t-tests. The probability of α-type errors is shown on the right.

4.1.3 Effect of Time on Neuronal Properties

Neurons in the non-injected control group were studied soon after injection of retrograde traces, whereas neurons in the sham control and experimental groups were studied at different periods over several weeks following the intramuscular injections of normal or acidic saline. In Figure 2, we have shown only one mean for each parameter for the non-injected group, while the data from the other groups has been presented according to the delay between the second injection into the muscles and the time of recording. Note that with the exception of action potential amplitude, the means of the non-injected control group were similar to those of the sham control group on days 1-2 after the second injection.

In the experimental and sham control groups, the amplitude of the action potential (Fig 2A), its half-width (Fig 2B), and the duration of after-hyperpolarization (Fig 2D) all decreased significantly over the course of the experiment (Two-way ANOVA, Table 3), while the after-polarization became deeper (Fig 2C), and there was a small but significant effect of time on mean input resistance (Fig 2E,) that was non-linear. However, there was no effect of treatment on these five parameters (Table 3). On the other hand, treatment did have a significant effect
(Table 3) on the seven parameters described in 4.1.1: RMP (Fig 2F), firing threshold (Fig 2G), threshold of inward (Fig 2H) and outward rectifications (Fig 2I), oscillation threshold (Fig 2J; Fig 3A) and amplitude (Fig 2K, Fig 3A) and burst threshold (Fig 2L). The differences between the two groups were clearly present within the first 8 hours after the second injection (days <1), and were generally maintained throughout the experiment (Fig 2F-2L). The effect of time on these parameters was relatively small, but was significant for all (Table 3) except outward rectification threshold (Fig 2F) and oscillation amplitude (Fig 2H). There were no significant interactions between group and time (Table 3).

Table 3 Experiment 1: Effects of group and time on neuronal properties of the Sham Control and Experiment Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Time</th>
<th>Group * Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Membrane Potential, mV</td>
<td>&lt;0.0001</td>
<td>0.002</td>
<td>0.034</td>
</tr>
<tr>
<td>Firing Threshold, mV</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.30</td>
</tr>
<tr>
<td>Input Resistance, MΩ</td>
<td>0.29</td>
<td>0.002</td>
<td>0.31</td>
</tr>
<tr>
<td>Action Potential Amplitude, mV</td>
<td>0.18</td>
<td>&lt;0.0001</td>
<td>0.44</td>
</tr>
<tr>
<td>Action Potential Half-Width, ms</td>
<td>0.47</td>
<td>&lt;0.0001</td>
<td>0.16</td>
</tr>
<tr>
<td>After Hyper-Polarization Duration, ms</td>
<td>0.11</td>
<td>&lt;0.0001</td>
<td>0.61</td>
</tr>
<tr>
<td>After Hyper-Polarization Amplitude, mV</td>
<td>0.93</td>
<td>&lt;0.0001</td>
<td>0.11</td>
</tr>
<tr>
<td>Inward Rectification Threshold, mV</td>
<td>&lt;0.0001</td>
<td>0.63</td>
<td>0.47</td>
</tr>
<tr>
<td>Outward Rectification Threshold, mV</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.28</td>
</tr>
<tr>
<td>Oscillation Threshold, mV</td>
<td>&lt;0.0001</td>
<td>0.003</td>
<td>0.43</td>
</tr>
<tr>
<td>Amplitude of Oscillation, mV</td>
<td>&lt;0.0001</td>
<td>0.19</td>
<td>0.14</td>
</tr>
<tr>
<td>Bursting Threshold, mV</td>
<td>&lt;0.0001</td>
<td>0.013</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Results of 2-way ANOVA analyses: probabilities of α-type errors
Figure 2

A  + Sham control
   O Experimental
   D Non-injected control

B  Action Potential
   Half Width (ms)

C  After Hyperpolarization

D  After Hyperpolarization
   Duration (ms)

E  Input Resistance
   (MO)

F  Resting Membrane Potential
   (mV)

G  Firing Threshold
   (mV)

H  Inward Rectification
   (mV)

I  Outward Rectification
   (mV)

J  Oscillation Threshold
   (mV)

K  Oscillation Amplitude
   (mV)

L  Resting Threshold
   (mV)
4.1.4 Patterns of Firing

Seventeen of the 167 cells (10%) of the Experimental group were spontaneously active at resting membrane potential, whereas none of the control cells were spontaneously active. This difference was highly significant ($X^2 = 16.9$, df=1, $p<0.0001$).

We classified firing patterns induced by a 1 s suprathreshold current injection, depolarization into four types: Adapting, Train, Train and Bursting, and Bursting (Figure 3B). Adapting neurons generated a single burst of spikes at the beginning of the step pulse that rapidly diminished in frequency, while Train neurons fired throughout the pulse. Bursting cells showed recurrent bursts of action potentials throughout the pulse, while a small number of neurons alternated between recurrent bursting and trains of spikes (Train and Bursting neurons). Bursting emerged from the high amplitude oscillations, as shown in the insert of Fig 3B.

The prevalence of these firing patterns in the control and experimental groups are given in Table 4. There was a significant between-group difference in the distribution of firing patterns ($X^2 = 8.53$, df=3, $p=0.035$). In particular, a significantly greater percentage of control neurons were of the Adapting type, and thus unable to sustain firing ($X^2 = 4.84$, df=1, $p=0.028$).
Table 4. Experiment 1: Firing patterns

<table>
<thead>
<tr>
<th>Firing Pattern</th>
<th>Control (n=198)</th>
<th>Experimental (n=167)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapting (A)</td>
<td>121 (61%)</td>
<td>83 (50%)</td>
</tr>
<tr>
<td>Train (T)</td>
<td>40 (20%)</td>
<td>36 (22%)</td>
</tr>
<tr>
<td>Bursting (B)</td>
<td>29 (15%)</td>
<td>35 (21%)</td>
</tr>
<tr>
<td>Train/Burst (TB)</td>
<td>8 (4%)</td>
<td>13 (8%)</td>
</tr>
<tr>
<td>A</td>
<td>121 (61%)</td>
<td>83 (50%)</td>
</tr>
<tr>
<td>T+B+TB</td>
<td>77 (39%)</td>
<td>84 (50%)</td>
</tr>
</tbody>
</table>

Comparison between the control (including the non-injected group) and experimental groups of the percentages of cells displaying one of the four firing patterns observed. There was a significant difference in distribution between groups when neurons were distributed into the four categories ($X^2 = 8.53$, df=3, p=0.035), and also when grouped into Adapting and Non-adapting (T+B+TB) ($X^2 = 4.84$, df=1, p=0.028).
Figure 3

A. CONTROL

-48mV
-41mV
-59mV
-65mV

B. ADAPTING

-64mV

EXPERIMENTAL

-46mV
-51mV
-58mV
-66mV

2mV
100ms
500pA

300pA
100pA

RMP

TRAIN

-56mV
0pA

-53mV

BURSTING

-55mV

TRAIN AND BURSTING
4.2 Experiment 2

Sixteen control neurons and 15 experimental neurons were recorded.

4.2.1 Between-Group Comparisons of Neuronal Properties

As in Experiment 1, a two-way ANOVA showed group assignment had a significant effect on RMP, firing threshold, thresholds for inward and outward rectification, oscillation threshold, oscillation amplitude and action potential half-width. There was also a significant interaction between group and day for action potential amplitude. T-tests showed that the first five variables were significantly lower from sham control values 2 days after the injection, but of these, only RMP, firing threshold and outward rectification threshold were significantly lower on the 1st day (Table 5). There was a non-significant tendency for the action potentials to be of lower amplitude and greater duration (half-width) 2 days post-injection.
Table 5. Experiment 2: Neuronal properties

<table>
<thead>
<tr>
<th></th>
<th>Sham Control 1-day Post-injection (n=6)</th>
<th>Experimental 1-day Post-injection (n=8)</th>
<th>Sham Control 2 days Post-injection (n=8)</th>
<th>Experimental 2 days Post-injection (n=9)</th>
<th>P Group</th>
<th>P Day</th>
<th>P Group * Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Membrane Potential, mV</td>
<td>-53.88 ± 0.97</td>
<td>-58.50 ± 1.12 (P=0.01)</td>
<td>-54.50 ± 0.82</td>
<td>-58.22 ± 0.78 (P=0.005)</td>
<td>0.0001</td>
<td>0.85</td>
<td>0.63</td>
</tr>
<tr>
<td>Firing Threshold, mV</td>
<td>-40.56 ± 2.85</td>
<td>-49.93 ± 1.36 (P=0.01)</td>
<td>-42.23 ± 1.95</td>
<td>-53.91 ± 1.05 (P=0.0003)</td>
<td>&lt;0.0001</td>
<td>0.17</td>
<td>0.56</td>
</tr>
<tr>
<td>Input Resistance, MΩ</td>
<td>79.37 ± 7.87</td>
<td>58.17 ± 5.99</td>
<td>67.25 ± 5.30</td>
<td>75.67 ± 11.01</td>
<td>0.46</td>
<td>0.75</td>
<td>0.093</td>
</tr>
<tr>
<td>Action Potential Amplitude, mV</td>
<td>87.95 ± 3.85</td>
<td>94.88 ± 5.15 (P=0.29)</td>
<td>88.02 ± 2.35</td>
<td>79.51 ± 3.62 (P=0.07)</td>
<td>0.84</td>
<td>0.051</td>
<td>0.049</td>
</tr>
<tr>
<td>Action Potential Half-Width, ms</td>
<td>0.83 ± 0.03</td>
<td>0.89 ± 0.05 (P=0.3)</td>
<td>0.81 ± 0.03</td>
<td>0.93 ± 0.05 (P=0.06)</td>
<td>0.037</td>
<td>0.81</td>
<td>0.48</td>
</tr>
<tr>
<td>After Hyper-Polarization Duration, ms</td>
<td>52.69 ± 9.13</td>
<td>46.77 ± 18.12</td>
<td>62.76 ± 4.15</td>
<td>43.91 ± 9.97</td>
<td>0.25</td>
<td>0.73</td>
<td>0.54</td>
</tr>
<tr>
<td>After Hyper-Polarization Amplitude, mV</td>
<td>9.16 ± 1.46</td>
<td>5.59 ± 1.13</td>
<td>9.28 ± 0.73</td>
<td>8.77 ± 0.99</td>
<td>0.080</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>Inward Rectification Threshold, mV</td>
<td>-64.13 ± 2.42</td>
<td>-67.67 ± 1.36 (P=0.23)</td>
<td>-62.63 ± 1.98</td>
<td>-73.11 ± 3.14 (P=0.014)</td>
<td>0.010</td>
<td>0.44</td>
<td>0.18</td>
</tr>
<tr>
<td>Outward Rectification Threshold, mV</td>
<td>-45.13 ± 3.00</td>
<td>-54.00 ± 3.29 (P=0.07)</td>
<td>-41.12 ± 3.92</td>
<td>-58.89 ± 2.35 (P=0.02)</td>
<td>0.0003</td>
<td>0.86</td>
<td>0.18</td>
</tr>
<tr>
<td>Oscillation Threshold, mV</td>
<td>-49.00 ± 3.70</td>
<td>-55.33 ± 1.48 (P=0.15)</td>
<td>-53.63 ± 1.24</td>
<td>-58.11 ± 1.21 (P=0.02)</td>
<td>0.023</td>
<td>0.11</td>
<td>0.68</td>
</tr>
<tr>
<td>Amplitude of Oscillation, mV</td>
<td>1.05 ± 0.12</td>
<td>1.20 ± 0.30 (P=0.66)</td>
<td>0.76 ± 0.08</td>
<td>2.01 ± 0.17 (P=0.0004)</td>
<td>0.0003</td>
<td>0.14</td>
<td>0.003</td>
</tr>
<tr>
<td>Bursting Threshold, mV</td>
<td>-30.25 ± 5.86</td>
<td>-39.33 ± 4.48</td>
<td>-41.00 ± 5.86</td>
<td>-45.25 ± 3.01</td>
<td>0.21</td>
<td>0.12</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Comparisons of mean ± SE of measurements made on cells one or two days after a single bilateral injection of normal (Sham Control) or acidic (Experimental) saline. Probabilities of α-type errors for the two-way ANOVA analyses and Ps for significant t-tests are shown.
5.0 DISCUSSION

We postulated that changes in muscle spindle phenotype could contribute to chronic muscle pain, and investigated the effects of algesic injections into muscle on the intrinsic properties of spindle afferents. There is evidence of participation of large myelinated primary afferent in the development of hyperalgesia. Vibration or light mechanical stimuli that activate large diameter skin afferents can produce pain after peripheral tissue or nerve injury. There is also evidence that as tissue and nerve damage occurs, oscillation and ectopic discharge increases in sensory neurons. Oscillations increased the probability of action potential discharges because when the amplitude of an oscillation sinusoid reaches threshold, action potentials are evoked. Our hypothesis is that under chronic muscle pain conditions, there is an increase in excitability of primary sensory afferents as well as ectopic firing. This increase in excitability can lead to the hyper-activation of pain pathways in a more robust way which can increase pain sensitization and ultimately activate other afferents and interneurons.

Behavioural changes were observed in the animals that received injections of acidic saline leading us to conclude that the animals were indeed experiencing pain. Animals injected with this algesic substance tended to rub their jaw more often and to have very minimal movements. They experienced sudden shakes and shivering which were not observed in the control group under similar conditions. Exploratory behaviours in new environments were also reduced in these animals.
Our data show that injections of acidic saline into the masseter muscle of rats appears to cause several changes in the electrical properties of the somata of muscle spindle afferents on the ipsilateral side. The changes began within 24 hours of the first injection and lasted for as long as 38 days after the second injection. They were present at all ages tested (P6- P44). Neurons innervating muscles injected with acid saline had a significantly lower mean RMP and a lower mean spike threshold. Inward and outward rectification occurred at more hyperpolarized potentials, as did high frequency membrane oscillations and spontaneous bursting. The maximum amplitude of the oscillations at a given membrane potential was also higher. Cells obtained from the pain group exhibit more bursting and train patterns of firing whereas those from the control seemed to adapt more. In this case more than half the recorded neurons from the pain groups combined had bursts and train firing patterns. This could potentially be explained by an increase in excitability caused by the algesic injection into the masseter muscle.

Some intrinsic properties remained unaffected. Acid saline had no effect on the input resistance and the current needed to drive the cell to its threshold potential. Also, we did not find any significant differences between the groups in the amplitude or half-width of the action potential, or on the after-hyperpolarization. It also had no effect on NVmes neurons on the contralateral side. Oscillations and repetitive firing capability depends on the activation of sodium and potassium conductance in the neurons (Liu et al. 2000). MesV spindle afferents have
resonant properties that produce high frequency oscillations which are sinusoidal fluctuations of the membrane potential and induce rhythmic bursting with depolarization. These properties are voltage-dependent and can be regulated by synaptic inputs (Verdier et al. 2004). It has been suggested that oscillations make a fundamental contribution to neuropathic dysesthesia and pain because their incidence increases in sensory neurons having their cell bodies in the dorsal root ganglia when nerve damage occurs (Liu et al. 2002). Axotomy induces changes in the neurochemical content of the mesencephalic trigeminal nucleus, as well as changes in the expression of many sodium and potassium channels (Liu et al. 2000). Since the increase in excitability was not associated to changes in the input resistance, we suggest that no major morphological changes occurred and that no new conductances appeared. The alteration observed could possibly be due to changes in voltage dependency of the 2 currents that play an important role in rectification.

We hypothesized that by exposing the terminals of the NVmes cells in the masseter muscle spindles to a low pH solution, we would see changes in the membrane properties and firing. NVmes neurons lack ASIC3 channels (Molliver et al. 2005), but isolated NVmes neurons do respond to an increase in [H+], which makes them different from the neurons of the Gasserian ganglion (Connor et al. 2005). Small changes in pH between 7.4 and 6.0 caused large changes in inward current in ganglion neurons but small changes in NVmes. However, further reductions in pH produced small currents in ganglion neurons, but large currents
in NVmes. This suggests that these highly specialized mechanoreceptors could be especially sensitive to low pH.

5.1 Acid Saline Induces Long-Lasting Pain and Hyperalgesia

Intramuscular injections or infusion of buffered hypertonic saline have been used to induce tonic pain in human muscles, including the masseter (Stohler 1992, Svensson et al. 1998). The pattern of radiation, pain quality and motor disorders are similar to those found in Temporomandibular Disorder patients with chronic myalgia (Svensson et al. 1998, Lund et al. 1991). However, symptoms resolve rapidly after the injection ends. Because deep-tissue hyperalgia seems to persist for much longer in humans if the pH of solution is reduced, Sluka et al. (2001) used solutions of low pH saline in an attempt to provide an animal model of muscle chronic hyperalgesia. They found that two 100µl injections (2 to 5 days apart) of saline at pH 4.0, into one gastrocnemius muscle of male Sprague-Dawley rats caused a bilateral hyperalgesia that lasted for 30 days, despite the fact that the injections caused no more visible tissue damage than injections of normal saline. They have shown that the hyperalgesia is absent in mice lacking ASIC3 acid sensing ion channels (Sluka et al. 2003), and have used the model to study the role of spinal excitatory amino-acid and opioid receptors in the maintenance and control of the hyperalgesia (Sluka et al. 2002; Skyba et al. 2005).
5.2 NVMes Neurons

The principal neurons of NVmes have large round or ovoid cell bodies with a single process that runs caudally in a well defined tract. Each stem axon gives rise to central branches and to a peripheral axon that innervates one of two types of sensory receptor: muscle spindles and presso-receptors in the periodontal ligaments (Cajal 1909, Corbin 1940). We are confident that all the neurons from which we recorded were muscle spindle afferents because they contained a fluorescent label that had previously been injected into the masseter muscle.

5.3 Spontaneous oscillations

Spontaneous oscillations in transmembrane voltage seem to be common in large-diameter primary afferent cell bodies. Puil et al. (1989) first described spontaneous oscillations and associated firing in trigeminal ganglion neurons in vitro. Amir et al. (1999) reported that 35% of large diameter DRG neurons afferents showed oscillations in the 88-195 Hz range when depolarized, and there are now several published descriptions of similar behavior in NVmes neurons. Pedroarena et al (1999) described the properties of NVmes cells that were chosen because they exhibited subthreshold oscillations in membrane potential either spontaneously (52%) or during current injection. Verdier et al. (2004) recorded from the cell bodies of masseter muscle spindle afferents of rats from 10 to 30
days old. They reported that about 12% of neurons that showed oscillations at RMP fired spontaneous. Unlike DRG cells, NVmes primary afferents receive synaptic inputs from several sources (see Verdier 2004 for original references), but oscillations are independent of synaptic inputs because they persist in calcium-free medium (Pedroarena et al. 1999; Wu et al. 2001; Verdier et al. 2004). Axotomy increases proportion of neurons with membrane oscillations and increases firing (Study and Kral 1996; Amir et al. 1999; Liu et al, 2000).

5.4 Burst Threshold and Firing

The changes in membrane properties and spontaneous oscillations brought about by the acid saline were accompanied by some spontaneous firing and significant increase in the prevalence of sustained firing during depolarization. An increase in spontaneous firing can occur in both the cell bodies of injured A fibers and intact C fibers (Wu et al. 2001, Boucher et al. 2000, Liu 2000); this spontaneous ectopic discharge following nerve injury has been noted in skeletal muscle afferents as well (Michaelis et al. 1995). Amir et al. (1999) have reported that DRG neurons that failed to show oscillations even when deeply depolarized were unable to generate continuous discharge, signifying the importance of the oscillatory process for repetitive firing in this type of neuron.
5.5 Functional Implications

Our hypothesis is that under chronic muscle pain conditions, there is an increase in excitability of primary sensory afferents as well as ectopic firing. This increase in excitability can lead to the hyper-activation of pain pathways in a more robust way which can increase pain sensitization and ultimately activate other afferents and interneurons.

Under normal conditions, large calibre afferents (Aα and Aβ fibres) that innervate the skin activate second order neurons whose discharges leads to a tactile sensation such as touch; On the other hand, pain is signalled by activity in C fibres as well as A fibres nociceptive afferents leading to the activation of the CNS (Beitel and Dubner 1961, Burgess and Perl 1967, Gybels et al. 1979, LaMotte and Campbell 1978, Perl 1968, Torebjörk and Ochoa 1980).

Weerakkody and colleagues (2001) showed that during vibration, painfully strong pressure becomes less painful in an unexercised muscle. What could be accountable for this observation? This brings our attention back to the “gate control” theory of pain which states that under normal conditions, inputs from large calibre afferent fibres inhibit nociceptive input by presynaptic inhibition and consequently causing a decrease in the pain sensation (Calvillo 1978, Melzack 1965). So, if this is the case, then how do muscle spindle afferents gain access to pain pathways? There exists a central synaptic link between the large diameter afferents and the nociceptors afferent fibres (Rudomin 1990, Schmidt 1971), containing at least one interneuron. Stimulation of low-threshold
mechanoreceptors causes primary afferent depolarization (PAD) of the nociceptive afferents (Cervero et al. 2003). Consequently, activation of low-threshold mechanoreceptors with Aβ afferents under normal conditions evokes pre-synaptic inhibition of nociceptive afferents, causing a decrease in the pain sensation (Calvillo 1978).

The interneurons responsible for PAD form axo-axonic synapses onto the central terminals of the nociceptor primary afferents, which are endowed with GABA\textsubscript{A} receptors (Cervero et al. 2003), causing GABA which usually has an inhibitory effect to be released into the synaptic cleft at the site of the nociceptors enabling the Chloride (Cl\textsuperscript{−}) channels coupled to the receptors to open. There is a high concentration of Cl\textsuperscript{−} within afferent neurons; consequently opening of these Cl\textsuperscript{−} channels causes an outflow of Cl\textsuperscript{−}, making the cell more positive and leading to membrane depolarization. This small membrane depolarization which is also referred to as the PAD generates a pre-synaptic inhibition (Cervero et al. 2003). But if this PAD is in fact strong enough, it will generate an action potential that can reinvade the synaptic terminal, which could ultimately reinforce the pain sensitization. Furthermore, there are reports of central changes occurring in the dorsal horn due to nerve injury (Cook et al. 1987, Wall and Devor 1983, Woolf 1983, Cervero and Laird 1996b, Treede et al. 1992) that strengthen the synaptic link between the central pain signalling pathways and low-threshold mechanoreceptors; though this possible reorganization in the CNS does not account for the fact that hyperalgesia has the potential to emerge within seconds
after injury (Cervero et al. 2003). The incredibly vibrant characteristic of these changes suggests that incoming activity from low-threshold afferents from an area of secondary hyperalgesia gains access to the nociceptive sensory channel leading to pain sensitization through mechanoreceptor (Campbell et al. 1988, Cervero et al. 2003). A key component of developing and sustaining secondary hyperalgesia is incoming nociceptive activity from an area of primary hyperalgesia (Dubner and Ruda i992).

Another step to a better understanding of this phenomenon is knowing what wide dynamic range (WDR) cells are and what role they play in this machinery. These relay cells have the potential to respond to inputs from both nociceptive and non-nociceptive receptors and are located in the superficial layers of the dorsal horn of the spinal and trigeminal cord. They are referred to as WDR to their nature of being able to respond to a wide range of inputs. It has been suggested that when the nociceptors are activated due to the presence of tissue injury, there is an increase of excitability in the presynaptic inhibitory interneurons mediating the effect of large-diameter mechanoreceptor afferent fibres on nociceptor afferent terminals (Cervero and Laird 1996b). Hence for in a situation such as muscle injury, which causes increase in the input of low-threshold mechanoreceptors afferents innervating the muscle could ultimately lead to the generation of pain.
In a damaged neuron, ectopic firing presents itself throughout and could be explained by the phenotypic changes in the nature and distribution of sodium and calcium channels. These changes may be contributing factors not only to spontaneous pain, but to central sensitization as well. Amir et al have reported that DRG neurons that failed to show oscillations even when deeply depolarized, were unable to generate continuous discharge, signifying the importance of the oscillatory process for repetitive firing in this type of neuron (Amir et al. 1999). Moreover, ectopic discharge may also cause and preserve “central sensitization”; this hyper-excitatory state of the dorsal horn in the CNS may pathologically enhance peripheral sensory input, underlying the cause of hyperalgesia and allodynia (Campbell et al. 1988, Woolf 1983, Devor and Seltzer 1999, Bennett 1994). It has been reported that by preventing spontaneous and ectopic discharge, both allodynia and hyperalgesia could be eliminated (Sheen and Chung 1993, Gracely et al. 1992).

If the hyper-excitatory state of the spindle afferents leads to an increase of afferent firing descending from the brain towards the periphery (antidromic firing), this could cause Glutamate to be released in the periphery. Nociceptors that have Glutamate receptors could be activated by this, carrying pain sensitizations back to the CNS.
5.6 Limitations of the Study

During the time when I was carrying out Experiment 1, I was not blind to treatment, but at that point, I still had no understanding of the significance of the parameters that were measured. I had a very limited knowledge of neuroscience, and just followed an established series of protocols. Once the analyses showed significant differences between experimental and control groups, Experiments 2 was conducted during which I was blind to treatment. Since very similar results were obtained in Experiment 2, the lack of blinding seems to have been unimportant.
6.0 REFERENCES


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114. Skyba DA, King EW, and Sluka KA. Effects of NMDA and non-NMDA ionotropic glutamate receptor antagonists on the development and maintenance of hyperalgesia induced by repeated intramuscular injection of acidic saline. Pain 2002;98:69-78.


7.0 LIST OF FIGURES:

Figure 1: Methods used to describe properties of neurons.
A: Hyperpolarizing and depolarizing current injections of 1 s duration were used to construct current voltage relationships. A cursor was used to measure the transmembrane voltage just before the end of the current step. B: The points of inflection on the I-V curve were taken as the thresholds for inward and outward rectification. C: These were also calculated from current ramps (10 s duration). D: Data from the depolarizing current pulses was also used to calculate firing threshold, action potential amplitude and halfwidth, afterhyperpolarization (AHP) amplitude and duration. E: Maximum oscillation amplitude (inset), and thresholds for high-frequency oscillations and for burst firing were also calculated.

Figure 2: Effects of time after injection on neuronal properties.
Means and standard errors are shown for control (filled circle) and experimental (open circle) neurons recorded at 7 time periods after the second intramuscular injection. Data for the unoperated control rats (open squares) is shown for comparison.

Figure 3: High frequency membrane oscillations and firing patterns.
Examples of recordings made 14 days after the second injection in control (left) and experimental groups showing the difference in oscillation amplitude at similar membrane potentials. RMP- resting membrane potential. The amount of current injected is given on the right of each trace.
8.0 LIST OF ABBREVIATIONS:

Gamma-Aminobutyric Acid (GABA)
Adenosine Triphosphate (ATP)
Persistant Sodium Current (I_{Nap})
Resting Membrane Potential (RMP)
After Hyperpolarization (AHP)
Dorsal Root Ganglia (DRG)
Central Nervous System (CNS)
Delayed Onset Muscle Soreness (DOMS)
Temporomandibular Disorder (TMD)