

Review Article

Repeated Muscle Injury as a Presumptive Trigger for Chronic Masticatory Muscle Pain

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skeletal muscles sustain a significant loss of maximal contractile force after injury, but terminally damaged fibers can eventually be replaced by the growth of new muscle (regeneration), with full restoration of contractile force over time. After a second injury, limb muscles exhibit a smaller reduction in maximal force and reduced inflammation compared with that after the initial injury (i.e., repeated bout effect). In contrast, masticatory muscles exhibit diminished regeneration and persistent fibrosis, after a single injury; following a second injury, plasma extravasation is greater than after a single injury and maximal force is decreased more than after the initial injury. Thus, masticatory muscles do not exhibit a repeated bout effect and are instead increasingly damaged by repeated injury. We propose that the impaired ability of masticatory muscles to regenerate contributes to chronic muscle pain by leading to an accumulation of tissue damage, fibrosis, and a persistent elevation and prolonged membrane translocation of nociceptive channels such as P2X₃ as well as enhanced expression of neuropeptides including CGRP within primary afferent neurons. These transformations prime primary afferent neurons for enhanced responsiveness upon subsequent injury thus triggering and/or exacerbating chronic muscle pain.

1. Introduction

Musculoskeletal pain is estimated to afflict 15% of the population, is one of the most frequent symptoms encountered by primary care providers [1, 2], and comprises a substantial portion of the total cost of illness [1–3]. Muscle pain is a prominent component in many musculoskeletal disorders, including low-back pain, tension-type headache, fibromyalgia and whiplash [4–6]. In the craniofacial region, temporomandibular disorders (TMD) affect 4–12% of the population (~35 million in the United States), with masticatory muscle pain being the most frequent (66%) patient complaint [7]. TMDs are often not restricted to the temporomandibular joint, but frequently include pain and tenderness of the masticatory muscles [4–6] designated as Group I in the Research Diagnostic Criteria for TMD [8]. It is estimated that one-half of TMD cases are these masticatory myalgias [9]. Patients with TMD frequently also have fibromyalgia [10–12], a musculoskeletal disorder characterized by widespread

musculoskeletal pain and diffuse muscle tenderness [11]. Approximately 2–5% of the population meet the diagnostic criteria for fibromyalgia [13, 14]. The similarities of TMD and fibromyalgia have led to speculation that these conditions may involve common mechanisms of muscle pain with different durations [15, 16]. While little is known about the mechanisms underlying muscle pain, available data indicate that the mechanisms underlying muscle pain differ from those underlying cutaneous or visceral pain (for review see [17, 18]).

In spite of the prevalence of muscle pain, current therapies for muscle pain are often ineffective and can even be dangerous [19]. For instance, NSAIDs and COX-2 drugs are no more effective than placebo in treating some types of muscle pain and have substantial risks [20–25]. Weak opioids (e.g., codeine, tramadol) do not alleviate pain produced by muscle injury [23, 26]. More powerful opioids such as hydrocodone, morphine, and oxycodone can reduce chronic pain, but have many deleterious effects [27–29]. Thus it is

important to understand the mechanisms of muscle pain in order to develop new, effective therapeutic strategies for muscle pain.

Pain resulting from muscle disorders can be persistent, although the mechanisms by which this chronic pain becomes established are not understood. Patients with TMD and fibromyalgia exhibit altered central nociceptive processing [30–33], which is hypothesized to be generated by a peripheral trigger [34]. Nociceptive input from muscle afferents is particularly potent at generating CNS wind-up [35]. Recent muscle pain studies support the involvement of peripheral stimuli in chronic muscle pain by demonstrating that enhanced central pain processing in fibromyalgia is maintained by muscle afferent input [36, 37]. We predict that comparable processes exist in muscle-based TMDs, given the similar characteristics of fibromyalgia and muscle-based TMDs [15, 16]. In this communication, we explore the potential for muscle injury to contribute to the triggering and/or maintenance of chronic pain.

2. Typical Experimental Methods of Producing Muscle Pain Do Not Accurately Model Muscle Pain

In spite of its prevalence, currently there are no widely accepted models of muscle pain, and most methods used to investigate muscle pain do not accurately reproduce the features of pain reported in humans suffering from muscle pain. For instance, injection of exogenous substances such as complete Freund's adjuvant (CFA) [38–40] has been used to evoke inflammatory muscle pain. However, CFA produces a massive inflammatory response with large intramuscular vacuoles and enormous inflammatory cell infiltration [38], characteristics that differ so dramatically from those reported in muscle pain patients [41] that adjuvant injection has only very limited relevance for studies of muscle pain. While injection of hypertonic saline produces sensations that mimic muscle pain [42, 43], hypertonic saline activates both muscle nociceptors [44, 45] and nonnociceptors [46, 47] and does not alter muscle lactate or PGE₂ as reported in muscle pain [45]. Acidic saline injected into limb muscles activates some muscle afferents [47] and produces hyperalgesia [48–50]. Although ASIC3 (acid-sensing ion channels) are present on craniofacial muscle afferents [51, 52], injection of acidic saline into the masseter does not produce hyperalgesia or alter calcitonin gene-related peptide (CGRP) and substance P expression [51]. Acid saline, therefore, is a valuable model for limb, but not craniofacial muscle pain. Injection of the polysaccharide carrageenan activates fine muscle afferents and produces hyperalgesia [53–55], but does not elevate intramuscular TNF- α [23]. Since TNF- α is elevated in TMD and fibromyalgia [56–58], carrageenan is not an accurate model for TMD and fibromyalgia. Mustard oil and capsaicin both inflame tissue including muscle [59–64]. Mustard oil acts via TRP (transient receptor potential channel) A1 channels on a subset of unmyelinated fibers [65] while capsaicin acts via TRPV1 channels on a subset of unmyelinated muscle afferents [66]. Since algogens do not activate

thinly myelinated muscle afferents, they do not activate the spectrum of afferent types that participate in muscle pain [67–69]. Endogenous substances have also been injected into muscles. Serotonin, bradykinin, ATP, TNF- α , and NGF activate a subpopulation of muscle afferents and produce pain [47, 52, 70–77]. Single substances (5-HT, bradykinin, etc.) interact with subsets of nociceptors providing insight into specific aspects of nociception; however, they cannot simulate muscle pain that involves multiple nociceptive channels and afferent types. Even if multiple algogens are used [73, 78], the environment at nerve terminals during pain cannot be reproduced due to differences in spatial application of the algogen, diffusion, and the fact that different mediators do not appear simultaneously in inflammation. For these reasons, we concentrate in this communication on data derived from eccentric (lengthening) muscle contractions, a noninvasive, physiologically relevant *in vivo* model of producing muscle pain and inflammation. This model of muscle strain injury incorporates movement and contraction, fundamental properties of muscle [79, 80].

When an activated muscle is forced to lengthen because the external load exceeds the tension generated by the muscle contraction, this is termed a lengthening 'eccentric' contraction. Although eccentric contractions require less energy, the force generated during a maximal eccentric contraction is about double the force developed during a maximal isometric contraction; therefore eccentric contractions are more likely to produce damage than either isometric or concentric (shortening) contractions. Using an animal model, forceful eccentric muscle contraction uniquely disrupts selected myofibers [79, 81], comparable to the selective myofiber damage in humans after eccentric exercise [82]. As few as 12 unaccustomed voluntary eccentric muscle contractions can produce muscle pain often referred to as delayed onset muscle soreness (DOMS) [83]. Furthermore, inflammation evoked by muscle contraction typically develops more slowly than the pulsatile inflammation produced by injection of algogens such as CFA, which evoke changes in neuropeptide mRNA within 30 min [38]. Eccentric muscle contractions can be produced in the laboratory setting by manually lengthening a muscle during electrical stimulation [84, 85]. Only a few contractions are needed to induce readily detectable muscle inflammation [84, 86]. Both voluntary and stimulus-induced eccentric muscle contraction as well as rapid muscle stretching damage a subpopulation of myofibers [81, 82, 87, 88], produce muscle pain and soreness [45, 85], evoke myonecrosis, induce inflammatory infiltration, elevate inflammatory proteins [81, 84, 89, 90], decrease muscle force and range of motion (for review [91]), and activate genes associated with muscle repair and apoptosis [92]. Intramuscular calcitonin gene related peptide (CGRP) and vascular endothelial growth factor (VEGF), a proangiogenic cytokine which increases after exercise [93–95], also increase after eccentric muscle contraction [81]. While in this paper we present data derived from eccentric contraction produced by muscle lengthening following supramaximal muscle contraction, comparable, but smaller effects are observed following submaximal eccentric contractions and behaviors such as downhill running [90, 96].

3. Injury Evokes Differential Effects in Muscles from Various Body Regions

Repair and regeneration in hindlimb muscle following injury involves activation of satellite cells within 24–48 hours [97]. These mononuclear cells are situated outside the sarcolemma, but inside the basement membrane of each muscle fiber. They are normally quiescent, however they are thought to become active with stimulation (e.g., injury). Under appropriate conditions, satellite cells develop into myoblasts, which fuse to form myotubes [98]. Myotubes can then repair, or even replace, damaged muscle fibers. It is generally hypothesized that satellite cells, after several rounds of proliferation, are a determinant factor in the functional recovery of muscle. Within 7–14 days following injury, myofibers are approaching normal size [99] and myofibers return to normal by 24 days [100]. Evidence indicates that the response to eccentric contraction differs between hindlimb and forelimb muscles. Blood creatine kinase levels and muscle soreness are reported to be greater following muscle damage to forelimb compared to hindlimb muscles [101]. Recovery of function after injury is also reported to be slower in forelimb versus hindlimb muscles [101]. In fact, when direct comparisons are made using similar indices of muscle damage, creatine kinase levels are greater after forelimb eccentric contraction and muscle recovery is longer for forelimb muscles [102].

Masticatory muscle responds very differently to injury than hindlimb muscle. Twelve days following muscle injury produced by a single crush or freezing injury, large areas of muscle exhibit minimal evidence of muscle regeneration [98]. Following a similar injury, hindlimb muscle shows centrally nucleated fibers (CNFs), indicative of regenerating muscle. At 19–21 days following injury, masseter muscle regeneration is still impaired and the masseter muscle exhibits extensive interstitial connective tissue. Even 45 days following a single injury, regeneration of the masseter muscle is less extensive than observed in hindlimb muscle 12 days after injury [98].

We have observed comparable findings after muscle injury produced by a single bout of eccentric muscle contractions [81]. Adult, male Sprague Dawley rats 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) and the Use Committee and the Committee for Research and Ethical Issues of the IASP. All laboratory procedures were reviewed and approved by the University of Maryland Animal Care and Use Committee and every effort was made to minimize any suffering. We first anesthetized the skin overlying the masseter muscle by applying a topical anesthetic (2.5% lidocaine, 2.5% prilocaine). We used a combination of lidocaine and procaine because this eutectic mixture has been shown to produce more effective cutaneous anesthesia in humans than either substance alone [103]. After two hours, when topical anesthesia was well established, rats were anesthetized with isoflurane. We have previously shown that lidocaine/prilocaine cream produces cutaneous anesthesia in the rat facial skin at this time

[104]. A rod coupled to a stepping motor and potentiometer was then positioned in the diastema of the mandible. To produce eccentric contraction of the masseter muscle, we used an established *in vivo* model previously described for the hindlimb [79, 80, 105]. The masseter was contracted by electrical stimulation (1 s trains, 100 Hz, 0.3 ms pulse at 0.3 Hz) using surface electrodes. Stimulation current was adjusted (5–12 mA) to produce a supramaximal muscle contraction. Neurogenic plasma extravasation was prevented by anesthetizing the skin overlying the muscle [81] and using a high-frequency stimulation regime, which does not activate group III and IV masseter muscle afferent axons [106]. Eccentric muscle contraction was produced by displacing the mandible 25 degrees of jaw opening at a rate of 0.6°/ms 150 milliseconds into a maximal muscle contraction. Mandibular displacement was produced using a stepping motor (1.8°/step NMB Technologies, Chatsworth, CA) controlled by a custom LabVIEW program (LabVIEW, version 8.5 National Instruments, Austin, TX). Muscle torque was measured using a torque sensor (model QWLC-8 M Sensotec, Columbus OH) and amplifier (model DV-05, Sensotec). Angular displacement of the mandible was monitored with a potentiometer. Displacement, angular position, and torque were synchronized using a custom LabVIEW program. Signals were sampled at 2 KHz using a 16-bit analog-to-digital converter (PCI-6221, National Instruments). The eccentric muscle contraction regime consisted of 5 sets of 15 eccentric muscle contractions (75 total contractions) with a five-minute rest between sets.

Muscle regeneration was not evident 32 days after one bout of eccentric contraction of the masseter muscle [107] and considerable fibrosis was present (Figure 1). These characteristics correspond to the impaired regeneration and extensive fibrosis evident for at least 45 days after crush or freeze injury to the masseter muscle [98]. In contrast to the masseter muscle, hindlimb muscles such as the tibialis anterior regenerate in 7–12 days after crush or freeze injury [98] and 5–14 days after eccentric muscle contraction [105, 108].

We operationally defined muscle injury as a loss in the ability of the muscle to produce force. Torque of a muscle is represented by the equation $T = F * d$, where T is torque, F is muscle force, and d is the moment arm of the muscle. Because we use a maximal tetanic contraction and we measured torque at a fixed position, our measure of torque ultimately reflects muscle force. Maximal contractile force is a strong indicator of the overall status of a muscle [109] and a reliable indicator of injury [110, 111]. Therefore, we investigated loss of maximal torque following injury in masticatory and hindlimb muscles. A variety of contraction schemes were tested, and we found that 60 masseter eccentric contractions (0.6°/ms) produce a 43% reduction in maximal torque measured at resting length (L_0) 10 minutes after contraction (Figure 2, arrow $n = 6$ rats). For the tibialis anterior muscle ($n = 25$), 150 eccentric contractions produced in an analogous manner resulted in a 41% deficit in maximal torque (Figure 2, asterisk [105]). Thus, to produce a comparable loss of isometric force following a single bout of eccentric muscle contraction, less than one-half as many

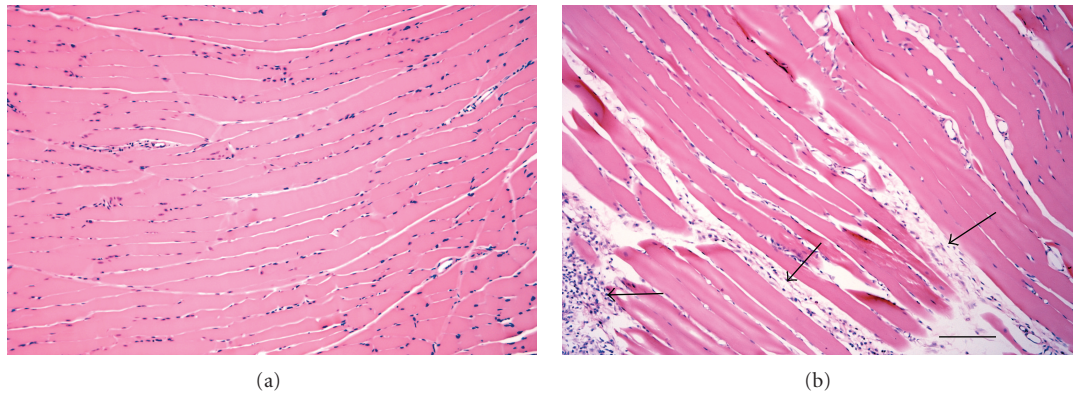


FIGURE 1: Photomicrograph of the masseter muscle stained with H&E. (a) Naive masseter muscle. (b) Masseter muscle 12 days after eccentric muscle contraction. Arrows indicate regions of fibrosis. Scale bar = 100 μm .

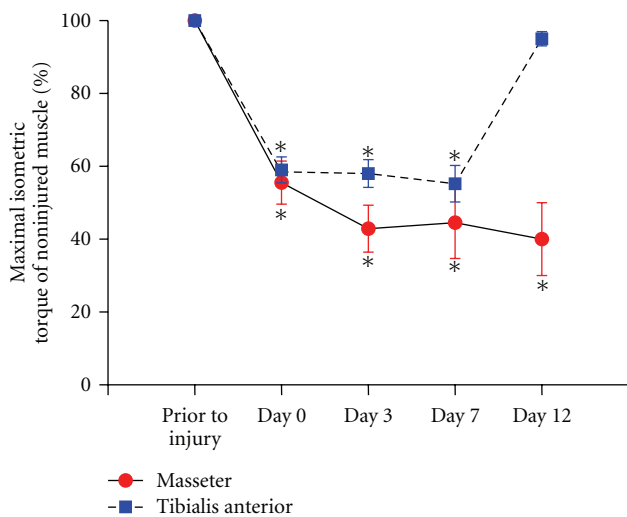


FIGURE 2: Maximal isometric torque produced before and at various times after eccentric muscle contraction. Solid line is masseter muscle and dashed line is tibialis anterior muscle. Note equivalent loss of force for Masseter and tibialis anterior at day 0 after eccentric contraction. Asterisks denote significant reductions from initial maximal torque. masseter = 60 eccentric contractions, tibialis anterior = 150 eccentric contractions, masseter $n = 6$, and tibialis anterior $n = 25$.

eccentric contractions of the masseter muscle were needed than for the tibialis anterior muscle (Figure 2). These data suggest that the loss of contractile force in the masseter after injury evoked by a single bout of eccentric muscle contractions is greater than in hindlimb muscles. Much less information is available on the effects of injury on muscles from other parts of the body such as the back and neck which may have profound significance for musculoskeletal pain disorders. It will be particularly important for future studies to determine the effects and functional significance of injury on muscles from other regions of the body, such as the back and neck, which may have profound significance for musculoskeletal pain disorders.

4. Masticatory Muscles Do Not Exhibit a Repeated Bout Effect

In limb muscles, lengthening contractions are associated with injury, but they can also provide significant protection against future injury. Compared to the first bout, a second bout of lengthening contractions in hindlimb and forelimb muscles is associated with a decreased loss of contractile force, less soreness, and a reduction in the amount of muscle proteins in the blood. However, little is known about the conditions that result in the protective adaptation [108, 112–117]. This adaptive effect is often referred to as the repeated bout effect (RBE) and has been demonstrated in both animals and humans (for review see [118]). While a number of mechanisms have been proposed to underlie the RBE including neuronal, cellular, and mechanical adaptations, the processes involved in the RBE are still not well established. Neuronal mechanisms, such as changes in motor unit recruitment, have been proposed. Although there is some evidence for changes in motor unit recruitment following injury, the RBE can be evoked by electrical stimulation [113], indicating that changes in motor unit recruitment alone are not sufficient to account for the repeated bout effect.

The RBE has also been attributed to cellular mechanisms, including change in the number of sarcomeres, excitation-contraction coupling, and/or inflammation. An increase in the number of sarcomeres has been reported following eccentric exercise [119–121]. However, the RBE can also be demonstrated following a minimal stimulus, such as a few eccentric contractions, or passive stretching, a stimulus that may be insufficient to evoke sarcomere remodeling [118]. While excitation-contraction coupling can be disrupted immediately following eccentric contraction [122], it does not correspond to the timing of loss of strength in humans several days following a repeated bout of eccentric contraction [112]. Inflammation typically occurs following eccentric muscle contraction [86, 90], and this inflammatory response is reduced following a subsequent bout of eccentric hindlimb or forelimb muscle contraction [114, 123]. It has been proposed that inflammation may help to provide a protective function from damage after subsequent bouts of

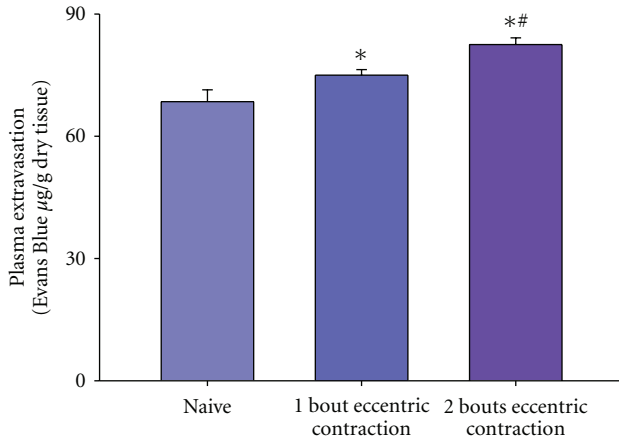


FIGURE 3: Plasma extravasation after one and two bouts of eccentric contraction of the masseter muscle. Note the increased plasma extravasation after two bouts of eccentric contraction. Asterisk denotes significant difference from naïve and number sign denotes significant difference from 1 bout.

eccentric muscle contraction [123, 124]. Myofiber damage produced by a bout of eccentric muscle contractions is reduced after subsequent bouts [125]. Thus, it is difficult to determine if the reduced inflammation that occurs following a repeated bout of contractions is a primary process, or reduced due to diminished tissue injury. However, it has been shown that passive stretching and concentric muscle contraction, processes that do not produce overt tissue damage evident at the light microscopical level, can evoke a small repeated bout effect [126]. Alteration in the mechanical properties of muscle including muscle stiffness and altered expression of cytoskeletal proteins have also been postulated to contribute to the RBE. While passive muscle stiffness increases following eccentric exercise [127], it is unclear that this increases the susceptibility of the muscle to injury [128]. Thus while several of these mechanisms may contribute to the RBE, the precise mechanisms of the repeated bout effect remain elusive.

Little is known about the effects of repeated injury on craniofacial muscle, therefore we have begun to examine repeated injury of the masseter muscle. Craniofacial muscle has distinct origins and developmental regulatory mechanisms from that of limb muscle. The masseter is derived from the first pharyngeal (branchial) arch and has been shown to respond differently to muscle injury [98]. The effect of impaired regeneration and fibrosis of the masseter muscle after repeated injury was initially investigated by examining plasma extravasation defined here as (wet muscle weight – dry muscle weight/wet muscle weight) \times 100, as an index of muscle edema [81]. Muscle edema significantly increased not only after one bout of eccentric contraction compared to naïve (Figure 3 asterisk) but also after two bouts of contraction spaced 12 days apart (Figure 3 number sign). Note that muscle edema increased significantly after two bout compared to one bout of contraction indicating a lack of repeated bout effect (naïve $n = 4$, 1 bout $n = 4$, 2 bouts

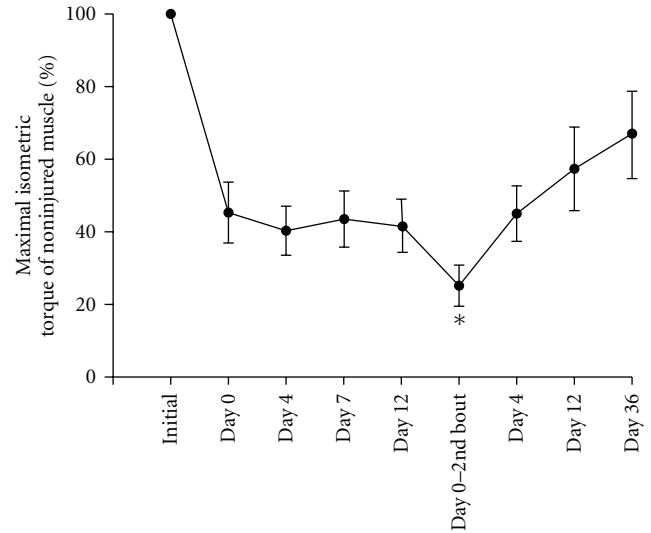


FIGURE 4: Effect of repeated masseter muscle injury on maximal isometric torque. Asterisk denotes significant difference from maximal torque after a single bout of eccentric contraction (day 0 initial bout).

$n = 6$, $P < .025$ for 2 bouts versus 1 bout and .049 for 1 bout versus naïve, ANOVA followed by Holm-Sidak method, Figure 3). Mechanical hyperalgesia was also measured by determining the threshold for a head withdrawal reflex [81]. Animals were initially habituated to stand unrestrained on their hindpaws and lean on the tester's hand covered with a leather glove. Mechanical thresholds were then determined by probing the masseter muscle through the facial skin using a rigid von Frey filament coupled with a force transducer with a fixed contact area (Electrovonfrey, model no 2290, IITC Inc). The force needed to produce a withdrawal of the head was recorded following five stimulus presentations at one minute intervals. The mean values of the five readings was used for analysis. Using this method, mechanical hyperalgesia was found to be more profound and persisted for at least 7–14 days longer after multiple bouts of eccentric contraction of the masseter muscle than one bout (ANOVA, $P < .05$, $n = 7$). Taken together, these data contrast strongly with data derived from hindlimb muscles, which show a RBE in regards to inflammation and muscle soreness [112, 113, 129]. We also examined the effects of two bouts of eccentric muscle contraction spaced 12 days apart on masseter contractile function by measuring torque in 7 male rats. After a second bout of eccentric contraction, masseter maximal torque decreased by 79% compared to the initial maximal torque at day 0, and decreased by an additional 60% compared to maximal torque immediately prior to the second bout of eccentric contractions (Figure 4). These data show that a second bout of eccentric contractions of the masseter muscle further reduces muscle force (Mann-Whitney rank sum test, $n = 7$ animals per group, $P = .026$) in contrast to the tibialis anterior muscle in which a second bout of eccentric muscle contraction results in very little or no further reduction in muscle force (i.e., repeated bout

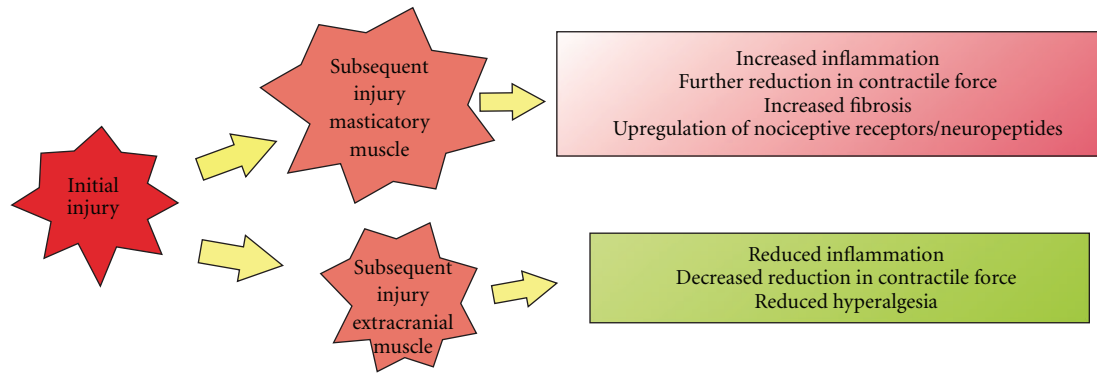


FIGURE 5: Diagrammatic representation of the response of masticatory versus limb muscle to repeated injury.

effect) [108, 113]. Thus, the masseter muscle not only lacks a repeated bout effect, but instead sustains increased damage upon repeated bouts of muscle injury (Figure 5). We propose this difference evokes mechanisms that contribute to chronic craniofacial muscle pain.

5. Synthesis of the Role of Muscle Injury in Chronic Muscle Pain Including Potential Therapeutic Targets

In this communication we show that repeated bouts of injury to masticatory muscles do not evoke the adaptive RBE present in limb muscles, but rather compound muscle injury. Patients with TMD and fibromyalgia exhibit altered central nociceptive processing [30–33, 37], which is most likely initially triggered from a peripheral source [34]. Nociceptive input from muscle afferent neurons is particularly potent at generating central nervous system wind-up [35]. One potential source for muscle injury is oral parafunctional behaviors. Evidence shows that oral parafunctional behaviors that increase muscle tension are good predictors of orofacial pain levels in patients with TMD [130, 131]. It is also known that experimental bruxism produces muscle soreness, described as moderate muscle pain that is exacerbated by movement [132, 133]. Horizontal jaw movement at 50% maximum voluntary contraction for 5 min, or jaw protrusion and retrusion under load, also produces delayed jaw muscle pain [134]. Finally, eccentric, but not concentric, contraction of the masseter muscle lowers the masseter pressure pain threshold [135, 136], which is consistent with the greater myofiber damage produced by eccentric contractions [137]. Since parafunctional behaviors can occur for prolonged periods, we propose that the impaired ability of masticatory muscles to regenerate results in neuronal transformations. This in turn chronically enhances the responsiveness of muscle primary afferent neurons to subsequent injury and thus serves as a source to initiate and/or exacerbate chronic muscle pain.

Nerve growth factor (NGF) is a homodimeric protein that binds to TrkA and p75 receptors [138, 139] and is implicated in mechanical and heat hyperalgesia [140–143]. Several findings establish a role for NGF in peripheral muscle

pain mechanisms. First, NGF levels in the masseter muscle of patients with TMD are negatively correlated with pressure pain threshold and positively correlated with descriptors of pain [144]. Levels of NGF are also elevated in inflamed muscle following injury including injury evoked by eccentric muscle contraction [145–147]. While other studies implicate NGF in muscle pain [77, 148, 149], high concentrations of NGF were used. Several sources for intramuscular NGF exist. Adult myofibers do not produce NGF [150], although developing and dystrophic myofibers can [150, 151]. Other potential sources for NGF include keratinocytes [152], fibroblasts [153], and mast cells [154–156]. NGF is known to upregulate many nociceptive channels and neuropeptides implicated in muscle pain [157–161], and, therefore, we propose that NGF is not only involved in acute muscle pain, but plays an instrumental role in chronic muscle pain by sustained modulation of nociceptive channel and neuropeptide expression. Among these, we expect that P2X₃ and CGRP are particularly important for deep tissue craniofacial pain.

P2X receptors comprise a family of ionotropic receptors which are activated by ATP (for review [162, 163]). In muscle, injection of ATP elicits pain [73] and activates muscle nociceptors [164]. Nonspecific P2X antagonists also reduce nocifensive behavior following muscle pain [165]. One member of the P2X family, the P2X₃ receptor, is specifically implicated in nociception [166]. Since a much higher percentage of craniofacial muscle afferent neurons express P2X₃ than limb muscle afferent neurons [167], P2X₃ is particularly implicated in craniofacial deep tissue pain. P2X₃ receptors are present on masseter muscle afferent neurons [168] and rapidly desensitizing currents characteristic of P2X₃ receptors can be activated in a subpopulation of masseter muscle afferents by applying ATP [52]. P2X₃ immunopositive muscle afferent neurons are increased 15 days following repetitive muscle contraction and rapid stretching [81]. Thus, physiologically relevant stimuli upregulate P2X₃ in primary muscle afferent neurons for prolonged periods of time. One potential source of ATP to activate P2X₃ receptors is ATP released from the cytosol of damaged cells. In coculture systems, action potentials and inward currents are evoked in nociceptors when nearby cells are mechanically damaged, and these responses are demonstrated to be

mediated by ATP [169]. In muscle, the concentration of ATP within myofibers is approximately 10 mM [170], a concentration that readily activates muscle primary afferent neurons *in vivo* [164], demonstrating that sufficient ATP is present within myofibers to activate muscle afferent neurons. Since eccentric muscle contraction mechanically damages myofibers and disrupts their membrane [81], we propose that ATP is released from damaged myofibers following muscle injury and activates P2X₃ receptors on muscle nociceptors.

Considerable evidence implicates the neuropeptide, calcitonin gene-related peptide (CGRP) in nociception and inflammation [171–175]. CGRP is a 37 amino acid neuropeptide synthesized in primary afferent neurons. CGRP is a potent vasodilator of blood vessels [171, 176] including those in muscles [176], and mediates neurogenic inflammation [177]. CGRP has been implicated specifically in nociceptive mechanisms from deep tissues [178], including muscle [54] and intramuscular CGRP is significantly increased following muscle injury evoked by eccentric muscle contractions [94]. Seventy-five percent of masseter P2X₃ muscle afferents colocalize CGRP [168]. We predict that this extensive colocalization indicates greater interaction between CGRP and P2X₃ in trigeminal, compared to dorsal root ganglion neurons, where neuropeptides and P2X₃ are segregated [179, 180]. NGF not only upregulates CGRP [181], but also P2X₃ [157, 159, 182]. We propose that increased intramuscular NGF following myofiber injury and muscle inflammation not only upregulates CGRP, but also increases P2X₃ expression in muscle primary afferent neurons priming the responsiveness of these neurons upon subsequent injury.

Additional factors to consider are that stress and autonomic dysfunction are correlated with some muscle pain disorders [183–187]. When stress is combined with eccentric contractions of hindlimb muscles, allodynia persists for up to 35 days and becomes bilateral [188]. This finding demonstrates that muscle injury can evoke long-lasting neuronal plasticity. Thus, we predict that acute muscle injury, particularly when combined with stress, can evoke central nervous system changes after which pain can become independent of peripheral drive, and that intermittent muscle injury exacerbates pain even after central pain transformations have occurred. Little is known about potential interactions between muscle injury, autonomic dysfunction, and the development of chronic muscle pain, making it an important area for future research.

We hypothesize that peripheral mechanisms involving primary afferent neurons from deep tissues are instrumental in the development of central nervous system transformations, such as central sensitization that occurs in muscle-based TMDs and fibromyalgia [30–32, 189]. Thus, agents capable of reducing primary afferent drive evoked by muscle inflammation have potential as acute therapeutics and as modulators of long-term nociceptive phenomena. We propose that increased intramuscular NGF after muscle injury plays a critical role in chronic pain by persistently upregulating P2X₃ and CGRP in muscle primary afferent neurons. Although selective P2X₃ antagonists exist [190], rather than directly targeting P2X₃, a potentially more

powerful approach is to concentrate on CGRP antagonists and NGF biologics (for review [191, 192]), because these agents have the potential to decrease both neurogenic inflammation and P2X₃ upregulation. We predict that CGRP antagonists will not only reduce vasodilatation and CGRP synthesis and release, but that they will also attenuate the upregulation of P2X₃ receptors, reducing the activation of muscle nociceptors by ATP. We also anticipate that anti-NGF antibodies will have multiple antinociceptive actions. These include, but are not limited to, blocking NGF-mediated upregulation of CGRP and reducing the upregulation of P2X₃ due to CGRP.

6. Conclusions

In this communication we have described differences in the response to injury of masticatory muscle versus hindlimb muscle. We also included new evidence that masticatory muscles do not adapt to repeated injury as occurs in hindlimb muscle (i.e., masticatory muscles do not exhibit the repeated bout effect). We propose that acute bouts of injury, as occurs during oral parafunctions, increase intramuscular nerve growth factor evoking a persistent upregulation of nociceptive receptors and neuropeptides. This mechanism primes primary afferent neurons for enhanced responsiveness upon subsequent injury and serves to trigger and/or exacerbate chronic muscle pain.

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