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The effects of adrenergic, serotonergic, and cholinergic modulation on hairy skin low threshold mechanoreceptors

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B.A., University of Hawaii at Hilo, 2013

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Abstract

The effects of adrenergic, serotonergic, and cholinergic modulation on hairy skin low threshold mechanoreceptors

By Makalele A. Provost

Low threshold mechanoreceptors (LTMRs) play a vital role in interactions with our physical environment. They convey information regarding gentle touch, movement across the skin, and hair follicle deflection, among other things. Perception of these types of stimuli can become disrupted in neuropathic pain states after spinal cord injury (SCI), particularly in allodynia, which is characterized by painful sensations in response to innocuous tactile stimuli. While central sensitization is known to play a role, alterations in peripheral sensory processing may also contribute. In particular, recent evidence has implicated C-LTMRs, which normally encode for pleasant touch, in the development and maintenance of allodynia. Several factors may contribute to altered peripheral processing after injury, including inflammation, sympathetic activity, and neuromodulation. The effects of neuromodulation on hairy skin LTMRs have not been thoroughly examined. This project set out to determine the effect of adrenergic, serotonergic, and cholinergic modulation on LTMR activity, with particular emphasis on C-LTMRs. Additionally, we examined the effect of SCI on C-LTMR response properties. We developed a novel electrophysiological recording setup and used an *in-vitro* skin-nerve preparation to record from dorsal cutaneous nerves while stimulating the outside of hairy skin in naïve, sham, and SCI mice. We used an optogenetic approach to selectively recruit C-LTMRs, and used puffs of air at calibrated forces to broadly recruit all hairy skin LTMRs. To assess neuromodulation, we bath-applied a reuptake or cholinesterase inhibitor, followed by norepinephrine, serotonin, and acetylcholine, respectively. Adrenergic modulation, including reuptake inhibition, decreased the response magnitude of optogenetically recruited C-LTMRs and mechanically recruited LTMRs. Serotonergic modulation had a similar effect in both stimulation paradigms. Cholinergic modulation did not significantly reduce the activity of C-LTMRs. Application of acetylcholine, but not the cholinesterase inhibitor, reduced the total response magnitude of LTMRs recruited at certain forces. These neuromodulators can be released in the skin via sympathetic efferents and immune cells, and it is well-known that SCI alters sympathetic drive and results in inflammation. Neuromodulation via inflammatory mediators or altered sympathetic output might constitute a mechanism by which altered signaling in the periphery contributes to maladaptive pain processing following spinal cord injury.

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Contents

Tables and Figures

Introduction

The purpose of this project is to explore the effects of cholinergic, adrenergic, and serotonergic modulation on peripheral sensory processing, with particular regard to how such modulation might contribute to altered signaling in hairy-skin low threshold mechanoreceptors (LTMRs) including in relation to possible contributions toward the development of maladaptive pain processing following spinal cord injury (SCI). The following sections will discuss (a) the various types of LTMRs present in hairy skin and their typical functions, with emphasis on C-LTMRs, (b) spinal cord injury and neuropathic pain, chiefly tactile allodynia, (c) drivers of altered peripheral sensory signaling, particularly sympathetic activity, inflammation, and neuromodulation, (d) sources and known actions of acetylcholine, norepinephrine, and serotonin in the skin, and (e) the role of C-LTMRs in tactile allodynia.

Mechanoreceptors

As the largest sensory organ, the skin is equipped with a wide array of specialized cutaneous sensory afferents. It is capable of detecting an impressive range of sensory modalities including thermosensation, pain, itch, and a variety of distinct mechanosensations such as indentation, vibration, stretch, and hair deflection. Perception and response to these stimuli is a key part of our interaction with the world, and is critical for survival. The afferents capable of detecting and coding these stimuli consist of morphologically and physiologically distinct classes of neurons which can all be classified as either Aβ, Aδ, or C fibers based on degree of myelination, axon diameter, conduction velocity, and cell-body size[1, 3]. Mechanosensitive afferents fall into two categories: High-threshold mechanoreceptors (HTMRs), which detect noxious or harmful mechanical stimuli, and low-threshold mechanoreceptors (LTMRs) that detect innocuous mechanical stimuli and occasionally respond to moderate changes in temperature[4]. Mechanoreceptors can be further classified by their recruitment thresholds and

adaptation properties [1]. In response to a sustained stimulus, mechanoreceptors can be slowly, intermediately, or rapidly adapting (SA, IA, and RA)[5]. Finally, these afferents are distinguished by the skin type that they innervate (hairy/glabrous) and their terminal endings (or the end-organs with which they associate). For the purposes of this thesis we will only be discussing low-threshold mechanoreceptors that innervate hairy skin. Table 1 lists the hairy-skin mechanoreceptor subtypes, their terminal endings, preferred stimulus, and recruitment thresholds. Figure 1 shows their morphology and location. The following sections will discuss the properties and functions of our mechanoreceptors of interest.

Receptor Subtype	Terminal ending/ end organ type	Location	Optimal Stimulus	Recruitment Threshold
Aβ SAI-LTMR	Merkel cell touch dome	Guard hair follicles	Indentation	>5 mN
Aβ RA-LTMR	Longitudinal lanceolate ending	Awl-Auchene/ Guard hair follicles	Skin movement Hair deflection	1 mN
Aδ-LTMRs	Longitudinal lanceolate ending	Awl-Auchene/ Zigzag hair follicles	Hair deflection	<1 mN
C-LTMRs	Longitudinal lanceolate ending	Awl-Auchene/ Zigzag hair follicles	Gentle, slow-moving touch	0.07 – 5 mN

Table 1. Properties of cutaneous low-threshold mechanoreceptors in hairy skin. Terminal ending/end organ, location, and optimal stimulus adapted from Abraira & Ginty, 2013[1]. Recruitment thresholds represent lowest force needed to elicit afferent firing. Adapted from Koltzenburg, Stucky, and Lewin 1997[2]

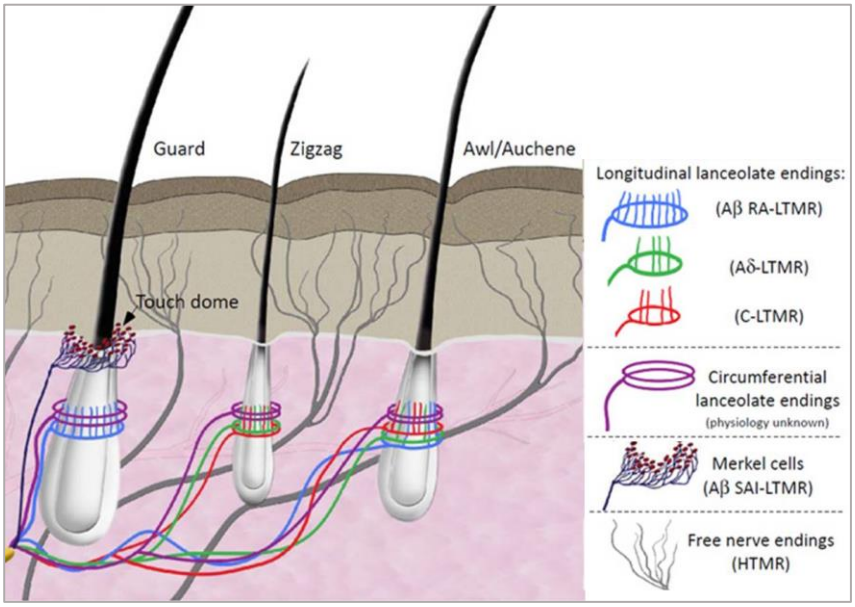


Figure 1. Organization of cutaneous afferents in hairy skin. C-, Aδ-, and Aβ RA-LTMRs form longitudinal lanceolate endings on the three types of hair follicles. Aβ SA1-LTMRs form merkel cell touch domes at the base of the hair shaft on guard hairs. HTMRs have free nerve endings that terminate in the upper layer of the dermis. Adapted from Abraira and Ginty, 2013[1].

Aβ- Low Threshold Mechanoreceptors

Aβ-LTMRs are thickly myelinated and have fast conduction velocities ranging from 16 to 96m/s[1]. The Aβ-LTMRs that innervate hairy skin can be divided into two types based on their terminal endings and adaptation characteristics: slowly adapting (SA) or rapidly adapting (RA). The slowly adapting Aβ SAI-LTMRs are associated with Merkel cell complexes (also known as "touch domes") and surround the base of guard hair follicles at the epidermal/dermal junction (Figure 1)[6]. In response to mechanical stimulation they exhibit sustained firing rates followed by irregular bursting which has been shown to correlate with indentation depths[7, 8]. The glabrous skin Aβ SAI-LTMR is thought to convey detailed information about object shape and texture[9], and while response properties of the glabrous and hairy skin types are similar, hairy skin is much less densely innervated by these afferents[8].

The second type of hairy-skin Aβ fiber is the rapidly adapting Aβ RA-LTMR. These afferents have been thoroughly characterized and their properties are relatively well conserved across species. They form longitudinal lanceolate endings with guard and awl/auchene hair follicles (Figure 1) and are uniquely tuned to detect hair follicle deflection[10]. They are not spontaneously active and they do not respond to changes in temperature. In response to hair follicle deflection they fire either only a few action potentials, or a burst of action potentials relative to the velocity and extent of deflection[1].

Aδ- Low Threshold Mechanoreceptors

Another type of mechanoreceptor innervating hairy skin is the Aδ-LTMR. These afferents are thinly myelinated and as such have conduction velocities that are over all slower than Aβ afferents, ranging from 5 to 30ms. Similar to the Aβ RA-LTMRs, they form longitudinal lanceolate endings, but selectively innervate only awl/auchene and zigzag hair follicles (Figure 1)[10]. They have exceptionally low mechanical thresholds and are maximally responsive to hair deflection, particularly deflection induced by gentle air puff stimulation. Additionally, they are

responsive to rapid cooling, but not warming, of the skin[4, 11]. Aδ-LTMRs have rapidly adapting response characteristics with bursts of action potentials at the start and end of sustained stimulation[1].

C- Low Threshold Mechanoreceptors.

C-LTMRs were discovered in animal hairy skin in 1939[12], but their existence in humans was not confirmed until the end of the century[13, 14]. In humans they are called C tactile (CT) afferents, but while the nomenclature may differ, the response properties of these afferents are conserved across species[14]. C-LTMRs are unmyelinated and have slow conduction velocities (0.2-2m/s)[1]. They respond to indentation forces below 5 mN, with some activation in response to forces as low as 0.07 mN[10]. C-LTMRs exhibit a U-shaped firing pattern in response to changes in stimulation velocity. Stimulation velocities in the range of 1-10 cm s^{-1} elicit maximal responses, and firing rates decrease with faster and slower movements[15]. In response to a maintained stimulus, they initially respond with a burst of firing, but then sustain a modest discharge[14]. Some afferents may generate after-discharges lasting up to several seconds following stimulus removal. C-LTMRs are highly fatigable in response to repeated application of identical stimuli[16]. Additionally, like Aδ-LTMRs, they display sensitivity to cooling, but not warming, of the skin[1].

While researchers have been steadily examining the electrophysiological properties of C-LTMRs, their anatomical features remained elusive due to lack of a unique molecular identifier. The first breakthrough came in 2006 when researchers identified a novel group of small diameter, unmyelinated dorsal root ganglia (DRG) neurons that expressed tyrosine hydroxylase (TH) and did not exhibit peptidergic phenotypes[17]. In 2009 Seal et al. discovered a subset of unmyelinated DRG neurons expressing the low abundance vesicular glutamate transporter VGLUT3. Direct recording from these $VGLUT3^{+}$ neurons identified them as C-LTMRs[18]. In

2011 a study utilizing intracellular recordings and immunohistochemical analysis identified a subgroup of C-fiber neurons with classically defined C-LTMR features, positive staining for TH, and expression of VGLUT3 mRNA[10]. This strengthened the previous evidence and opened the way for genetic labeling strategies. C-LTMR peripheral endings form longitudinal lanceolate endings with zigzag and awl/auchene hair follicles, but not guard hair follicles (Figure 1). A single murine C-LTMR arborizes and associates with approximately 18 hair follicles, forming a receptive field of 0.2-0.4 mm. Consistent with physiological recordings, glabrous skin is devoid of the peripheral endings of C-LTMRs[1]. In DRGs innervating the trunk and proximal limbs, C-LTMRs comprised more than 15% of neurons, while significantly fewer were found in DRGs that innervate distal limbs[10]. C-LTMRs selectively innervate lamina II of the dorsal horn (Figure 1)[1], and send projections to wide dynamic-range (WDR) spinoparabrachial neurons in lamina I[19].

C-LTMRs and the affective touch hypothesis.

The investigation of C-LTMR characteristics suggests that they play a distinct role from Aβ- or Aδ-LTMRs. The first evidence to support this supposition came from two patients who were selectively lacking Aβ afferents but had intact C fibers[20]. These patients were able to detect soft brush stroking and low-force monofilament indentation in areas of known CT afferent innervation. Importantly, they were unable to detect the same stimuli when applied to glabrous skin of the hand[21] which is devoid of C-LTMR innervation. While unable to give a precise description of the sensations elicited by soft stroking, both patients independently reported that it was a pleasant touch experience devoid of pain, tickle, or itch[22]. Studies using functional magnetic resonance imaging (fMRI) of cortical activation have given additional insight into the function of CT afferents. In healthy subjects soft brush stroking activates the somatosensory cortex as well as the insula – a region associated with motivation and feelings. In the patients lacking Aβ-LTMRs, the same stimuli activated only the insular region. Additionally,

greater activation of the posterior insular cortex and orbitofrontal cortex is seen following soft brush stroking of the forearm versus palm, while stroking of the palm versus forearm significantly activates somatosensory cortices[23]. In psychophysical experiments emotional descriptors are rated higher on the forearm while sensory discrimination is rated higher on the palm. Additionally, soft stroking in the same velocity known to preferentially activate C-LTMRs is reported as more pleasant that faster or slower stroking. In fact, there is a strong correlation between pleasantness ratings and CT firing frequency in response to soft stroking[20]. Furthermore, researchers used pharmacogenetic activation of C-LTMRs *in-vivo* to promote conditioned place preference in awake behaving animals, further supporting their role as pleasant, reinforcing afferents[24]. Taken together, this evidence has led to the CT affective touch hypothesis, which posits that C-LTMRs are uniquely tuned to code for the hedonic value of touch.

Spinal cord injury and neuropathic pain.

There are approximately 300,000 individuals with spinal cord injuries (SCI) in the US, and that number increases each year by more than 17,000[25]. SCI is a devastating event which can result in motor deficits below the level of injury, as well as perturbations in sympathetic function, and chronic pain syndromes below, at, or above the level of injury. Neuropathic pain is one of the most common SCI-induced pain syndromes. It is characterized by spontaneously occurring pain in the absence of stimuli, and by evoked pain in the forms of hyperalgesia (an exaggerated pain response) and allodynia (the sensation of pain in response to non-noxious stimuli)[26]. As many as 50% of SCI patients develop neuropathic pain[27], and it can so drastically impair quality of life that depression and suicide frequently occur[28]. Neuropathic pain is typically refractory to treatment despite advancements in pharmacological, surgical, and behavioral therapeutic strategies[29].

Understanding the mechanisms which contribute to neuropathic pain is particularly difficult given the vast number of anatomical, neurochemical, and inflammatory alterations which occur following SCI[30]. These changes have predominantly been documented within the spinal cord (e.g.[31-36]). Thus, central sensitization, in which dorsal horn neurons display persistent hyperexcitability after injury[37], has become the leading explanation for altered pain processing following SCI. Although the majority of neuropathic pain studies have demonstrated central mechanisms, a few have also documented a role for peripheral sensitization (see review: D'angelo 2013[38]). In 2009 Carlton et al. [39] discovered sensitized nociceptors in the rat forelimb following a T10 contusion SCI. These nociceptive afferents displayed three aberrant behaviors: increased background activity, lowered thresholds, and enhanced discharge rates. In 2010 Bedi et al.[40] found that dorsal root ganglia nociceptors exhibited SCI-induced spontaneous activity, even after dissociation from the spinal cord. These results suggest that altered pain processing following SCI may be due to both central and peripheral mechanisms. Potential mechanisms that may contribute to altered peripheral sensory signaling are discussed below.

Altered peripheral sensory signaling

In order to consistently perceive their preferred stimuli and encode those stimuli into meaningful sensations for the organism, sensory afferents have conserved characteristics. These include (a) preferred stimuli, (b) conduction velocity, (c) the threshold at which mechanical stimulation elicits firing, (d) firing properties in response to sustained stimulation such as adaptation and fatigability, (e) waveform of the evoked action potential, (f) location and shape of terminal endings, (g) receptive field size and shape, and (h) projection and synaptic location within the spinal cord. Alterations in central responses can be attributed to changes in any number of these afferent characteristics. Furthermore, external factors that may drive those

changes are numerous. Several potential drivers of altered peripheral signaling are discussed below:

Sympathetic activity

Cutaneous sympathetic efferents innervate many components of the skin including blood vessels, erector-pili muscles, and hair follicles[41]. The mechanism of action of postganglionic autonomic nerves in the skin is predominantly via release of norepinephrine[42]. Along with many other actions, adrenergic sympathetic fibers have been shown to influence the activity of various mechanoreceptors. In the rat, sympathetic stimulation resulted in frequency-dependent suppression or facilitation of cold-sensitive units[43]. In the cat, stimulation of sympathetic fibers was shown to increase firing in Aβ SA-LTMRs, and this effect did not appear to be related to pilomotor or arterial changes[44]. In 1982 Roberts and Levitt demonstrated that electrical stimulation of truncal sympathetic fibers reduced the recruitment thresholds of hair receptor units to mechanical stimulation. Furthermore, they confirmed that sympathetic fibers are spatially associated with hair receptor afferents, suggesting that sympathetic modulation of mechanoreceptors is due to direct neurotransmitter release[45]. A few years later C-LTMRs specifically were shown to increase their activity following sympathetic stimulation in both rabbits and cats, and changes due to blood flow or temperature were ruled out[46, 47]. Conversely, in a recent study investigators found that sympathetic stimulation in humans resulted in only moderate changes in mechanoreceptor activity and these effects were likely due to secondary mechanisms such as blood flow[48]. However, these recordings were conducted only in glabrous skin mechanoceptors, while the previously reported direct sympathetic innervation of cutaneous afferents was at hair follicles.

The sympathetic nervous system is widely considered to be involved in several pain disorders, including neuropathic pain and mechanical allodynia[49]. Interestingly, studies in both humans and animals have found no correlation between sympathetic stimulation and the activity of nociceptors[47, 50]. Given the evidence for sympathetic actions on low-threshold, but

not nociceptive, afferents they represent a likely population that could facilitate the relationship between the sympathetic nervous system and neuropathic pain. It is well established that both sympathetic disruption and neuropathic pain are common outcomes of spinal cord injury[26, 38, 51-53]. Given that (a) SCI alters sympathetic activity and results in neuropathic pain, (b) sympathetic output alters recruitment and firing patterns of hairy-skin LTMRs via direct mechanisms, and (c) LTMRs have been implicated in the development of mechanical allodynia, it is possible that autonomic dysfunction following SCI may lead to changes in the firing properties of sympathetic efferent actions on LTMR afferent activity patterns that lead to central changes in spinal cord function consistent with the production of neuropathic pain.

Inflammation

Cutaneous inflammation is another mechanism by which peripheral signaling can be altered. There are known interactions **between** nerve fibers, cutaneous cells, and immune cells[54]. Furthermore, it has been well established that inflammatory agents increase the excitability and lower thresholds of nociceptive afferents[55-58]. Additionally, studies have identified a novel class of unmyelinated cutaneous afferents that are unresponsive to mechanical, thermal, and nociceptive stimulation, but become active in response to inflammatory processes[56, 59-61]. In rats with inflammation-induced cold allodynia, C-LTMRs showed increased activation in response to moderate cooling of the skin[62]. It is clear that inflammation can modify the signaling of cutaneous afferents, although to our knowledge there is little research with specific regard to low-threshold mechanoreceptors beyond the one mentioned above.

Neuromodulation

Beyond direct mechanical stimulation, afferent signaling can be driven by a number of mediators including neuropeptides. Sources of these neuropeptides are wide-ranging and include postganglionic autonomic fibers, epithelial cells, immune cells, and other cutaneous

sensory fibers. Acetylcholine is released in the periphery by keratinocytes, lymphocytes, and melanocytes[42, 63]. Sympathetic fibers release norepinephrine (NE), predominantly at blood vessels for vasoconstriction[63]; Although, as mentioned previously, sympathetic fibers co-innervate hair follicles along with LTMRs, suggesting a route for direct adrenergic modulation of those primary afferents[45]. Merkel cells (MCs), a type of mechanosensitive epidermal cell, represent another method of neuropeptide-modulated tactile signaling. The specific type of chemical neurotransmission used by these cells is still a subject of debate. A 2016 study found that tactile stimulation of mouse whiskers resulted in vesicular serotonin release from Merkel cells at hair follicles; Furthermore, Aβ whisker afferents express 5-HT receptors and are activated following MC serotonin release[64]. A subsequent study used RNA sequencing and found that Merkel discs in touch domes express presynaptic molecules and machinery for adrenergic signaling[65]. These results confirm that MCs have neuromodulatory effects on cutaneous sensory afferents, although it seems that neurotransmission is via adrenergic mechanisms in glabrous skin, and serotonergic mechanisms in hairy skin. It is clear that there are many routes by which neurotransmitters, specifically acetylcholine, norepinephrine, and serotonin, are released into the skin milieu and may affect peripheral signaling. These three neurotransmitters and their known actions in the skin are discussed below:

Acetylcholine

Sources of acetylcholine in the periphery include keratinocytes, lymphocytes, and melanocytes[42, 63]. Acetylcholine exerts it actions on neurons that express either muscarinic or nicotinic acetylcholine receptors. Electrophysiological studies have also demonstrated that acetylcholine altered the activity of C-nociceptors and may be involved in the mediation of pain[66, 67]. To determine the specific actions of acetylcholine on C-nociceptors, researchers utilized selective muscarinic or nicotinic agonists and antagonists and determined that the two have opposing actions: nicotinic modulation was excitatory, while muscarinic actions caused

desensitization[68]. Further research confirmed that nociceptive C-fibers express the muscarinic receptor subtype M2[69]. It is unknown whether cholinergic receptors exist on other mechanoreceptors, although one study found that the acetylcholine analogue carbachol did not induce changes in Aβ- or Aδ-LTMR activity[70], suggesting that these fibers do not express cholinergic receptors. To our knowledge the actions of acetylcholine on LTMRs beyond this study remains unexplored.

Norepinephrine

Norepinephrine is secreted in the periphery by sympathetic free nerve endings, keratinocytes, melanocytes, and Merkel cells [63, 65, 71-74]. Adrenergic modulation plays a key role in anti-inflammatory processes. Adrenergic β-receptors are expressed on immune cells in the skin, and β-adrenoreceptor agonists have been shown to inhibit the release of pro-inflammatory TNF-α[75]. In the periphery adrenergic processes are also necessary for the progression of the hair growth[76]. Whether or not norepinephrine influences the activity of LTMRs, other than the one experiment involving C-LTMRs, has not been studied, but it is clear that it is present at hair follicles and therefore it has the potential to influence peripheral signaling.

Serotonin

As discussed previously, one mechanism of serotonergic modulation of afferent activity in the skin is via release from Merkel cells, specifically those at hair follicles[64]. Further sources of cutaneous serotonin include mast cells (although this result has only been demonstrated in rodents, not humans[77]), and melanocytes[78]. Changes in serotonergic signaling in the periphery have been shown to induce changes in sensory activity, namely in nociceptive signaling of C-fibers[79]. Furthermore, the role of serotonin in inflammatory processes is well documented[80, 81], and it can act as a pro-inflammatory agent, contributing to inflammation-

driven hypersensitivity and hyperalgesia after nerve injury[82, 83]. Unlike its role in nociceptive C-fiber modulation, the effect of serotonin on non-nociceptive low threshold mechanoreceptors is not well understood.

Altered C-LTMR activity and tactile allodynia

Given the abundant evidence for C-LTMRs in encoding normally pleasant stimuli, it seems surprising that they may be involved in the generation of pain in the form of tactile allodynia. Nonetheless, several studies have demonstrated a critical role for C-LTMRs and CT afferents in allodynia[18, 84-88]. The first to do so utilized a VGLUT3 knock-out mouse. Loss of VGLUT3, which has been established as a marker for C-LTMRs, impaired mechanical hypersensitivity to normally innocuous stimuli after injury[18]. A subsequent study identified TAFA4, a chemokine-like secreted protein, as an additional marker for C-LTMRs. Following chronic nerve constriction and carrageenan-induced inflammation, TAFA4-null mice exhibited enhanced mechanical hypersensitivity and increased excitability of dorsal horn lamina II neurons. Interestingly, this effect could be reversed via application of the TAFA4 protein[88]. The authors propose a scenario in which C-LTMRs release both glutamate and TAFA4, thereby promoting or preventing hypersensitivity, respectively. Furthermore, the projection to lamina I spinoparabrachial WDR neurons could serve as a possible anatomical pathway for C-LTMR mediated tactile allodynia[19]. Overall, there is sufficient evidence to implicate C-LTMRs in the development and maintenance of tactile allodynia, although none of these studies have been conducted using SCI-induced allodynia. The precise alterations of C-LTMRs following SCI have not been studied.

Methods

Transgenic Mouse Models

For the selective optogenetic stimulation of C-LTMRs, we used tamoxifen-inducible TH-Cre-ERt2::R26-ChR2-eYFP mice. Two lines of TH-Cre (JAX #s 008532 and 025614) mice were crossed with ChR2-eYFP (JAX # 012569) to create the transgenic line. When treated with tamoxifen, these mice, in a dose-dependent manner, express the channel rhodopsin-eYFP protein in TH^+ cutaneous afferent fibers. All experiments were conducted in naïve, sham-injured, or contusion spinal cord injured animals. Naïve animals were from the JAX # 008532 line, while sham and SCI animals were from JAX # 025614 line. Table 1 lists the animals used.

Treatment	Days Post-op	Genetic Line	TH-Cre Jax #	Experimental Paradigm	Cholinergic Modulation
Naïve (N=8)	N/A	TH:TdTomato	008532	Histology	N/A
Naïve (N=4)	N/A	TH:Chr2-eYFP	008532	Optogenetic & Mechanical	Neostigmine, Ach 1/10/100μM
SCI (N=1)	84	TH:Chr2-eYFP	008532	Mechanical	Epibatidine, Ach 1/10/100μM
SCI (N=4)	98, 97, 93, 95	TH:Chr2-eYFP	025614	Optogenetic & Mechanical	Epibatidine, Neostigmine, Ach 10μM
Sham (N=2)	100, 98	TH:Chr2-eYFP	025614	Optogenetic & Mechanical	Epibatidine, Neostigmine, Ach 10μM

Table 2. A total of 19 animals were used for these experiments. Eight naïve animals were used to determine appropriate tamoxifen dose, and to confirm expression in TH+ cutaneous neurons. Two independent TH-Cre lines were used and crossed with a ChR2-eYFP line to create our genetic models. In one animal the channel rhodopsin expression was unsuccessful, thus they were only used for the mechanical stimulation paradigm. The cholinergic protocol was altered across groups (namely the addition of the selective muscarinic agonist epibatidine).

Tamoxifen treatment

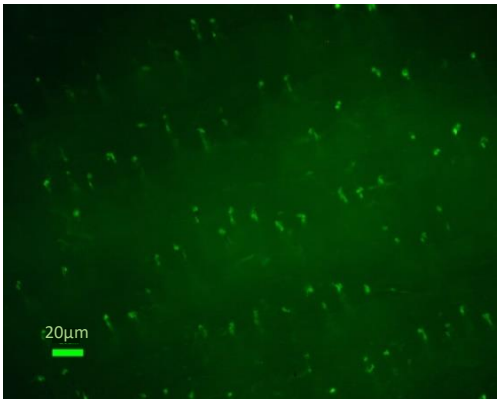
Following the protocol established by Li and Ginty (2014)[89] the animals received two days of tamoxifen treatment via subcutaneous injection. Briefly, to achieve maximal expression the animal is given a subcutaneous injection of 2mg tamoxifen, suspended in peanut oil, on their right flank at P20. Two days later they receive a second injection of 2 mg tamoxifen on their left

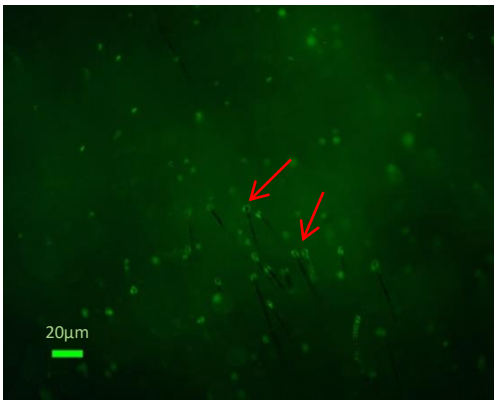
flank for a total of 4 mg tamoxifen. In older animals the dose was increased linearly such that a P30 animal would receive 5 mg total, P40 animals received 6 mg total, etc. The dosage amount needed to achieve robust expression was determined via immunohistological processing in TH-Cre-ERt2::TdTomato mice treated with varying doses of tamoxifen at different ages.

Immunohistological Processing

While TH-Cre mice were crossed with Cre -dependent ChR2-yellow fluorescent protein (YFP) mice in studies assessing their selective optogenetic recruitment described below, I first tested for selective Cre recombination in TH-Cre mice crossed with Cre -dependent TdTomato reporter mice. I processed the trunk skin of tamoxifen treated mice. The immunohistological protocol used was adapted from Li et al. (2011)[10]. Hairy skin comprising the animals' entire trunk area was dissected and cut along the dorsal and ventral midlines and subdermal connective tissue was removed. Small incisions were made along the rostral edge to identify orientation as well as to distinguish skin from the animals' left versus right. The skin was then treated with commercial hair remover, wiped clean with tissue paper, and remaining hairs were removed with tweezers. The skin was fixed in 4% PFA in PBS at 4^{o}C for 2 hours then transferred to PBS. The tissue was then washed with PBS containing 0.3% Triton X-100 (0.3% PBST) for 30 minutes and repeated ten times. Following PBST washes the skin was incubated with primary antibodies in 0.3% PBST containing 5% donkey serum and 20% DMSO at room temperature on a shaker for 3 to 5 days. Tissue was again washed in 0.3% PBST for 30 minutes, ten times. Skin was then incubated in secondary antibodies in 0.3% PBST containing 5% donkey serum and 20% DMSO at room temperature, on a shaker, in the dark for 2 to 4 days. Control tissue was incubated in 0.3% PBST containing 5% donkey serum and 20% DMSO at room temperature for 2 to 4 days with no secondary antibody. Following secondary incubation the tissue was again washed in 0.3% PBST for 30 minutes, ten times. It was then dehydrated in 50% methanol for 5 minutes and 100% methanol for 20 minutes, three times and finally cleared in BABB (Benzyl

Alcohol, sigma 305197-1L; Benzyl Benzoate, sigma B-6630; 1:2) at room temperature for 20 minutes. Tissue was then mounted on microscope slides with D.P.X. mounting medium, coverslipped, and allowed to dry at room temperature in the dark. A Nikon Eclipse E800 microscope and the program HCImageLive were used to image the tissue. Selective Cre recombination in TH+ C-LTMRs TdTomato reporter expression was confirmed via visual assessment of labeled longitudinal lanceolate endings surrounding hair follicles (Figure 2).





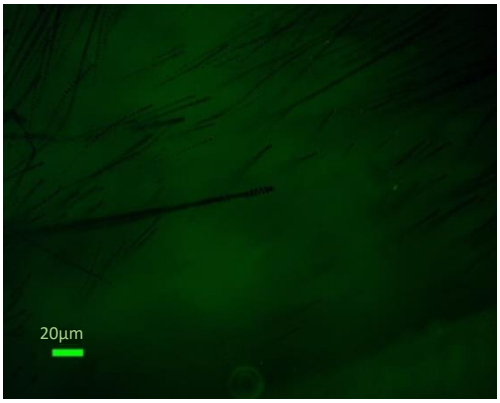


Figure 2. Cre-mediated expression of tdTomato reporter genes in TH+ C-LTMRs. Staining from back skin of TH-Cre-ERt2::TdTomato animal treated with 2mg/day tamoxifen at P30 and P32 **(A)** Terminal endings of C-LTMRs at hair follicles with hair removed. **(B)** Red arrows indicate terminal endings of C-LTMRs wrapping around base of a few remaining hairs. **(C)** Negative control from same animal shows hair follicles with no label.

Contusion SCI

Studies were conducted in three groups of animals: Naïve, sham-injured, and contusion-injured animals. Female and male adult mice (aged approximately P40 – P60) were deeply anesthetized with isoflurane. We performed a skin incision and dorsal laminectomy to expose the spinal cord. Contusion SCI were made at the dorsal surface of the spinal cord at level T10 with the Infinite Horizon impactor (Precision Systems and Instrumentation LLC, Lexington,

KY), at forces ranging from 50-70 kdynes, which is considered to be a moderate injury[90]. Bruising on the exposed spinal cord was used as an indicator of successful contusion injury. While no single experimental model of SCI reflects the complex heterogeneity of clinical SCI phenotypes, contusion injuries most closely mimic clinical symptomology[91]. Contusion injuries at the T10 spinal level have consistently been shown to lead to the development of neuropathic pain[90-93]. Sham injured animals underwent identical surgical procedures without the contusion injury. The elapsed time between surgical intervention and experimentation ranged from 75- 100 days (see Table 2).

Skin-nerve preparation

We developed a novel *ex-vivo* skin-nerve preparation to determine the changes in electrophysiological properties of C-LTMRs following SCI. The preparation consists of an 8x5 cm section of thoracic skin with attached dorsal nerves T8-T12. The area of detached skin comprises the entire trunk area of the mouse and covers the dermatomes associated with spinal cord segments T8-T12. To remove the skin an incision is made along the extent of the ventral midline. Incisions are then made just caudal to the forelimbs and rostral to the hindlimbs aiming toward the dorsal midline. The skin is pinned back onto Sylgard (Dow Chemical Company, Midland, MI), and connective tissue is removed to expose the dorsal nerves. The nerves T8 through T12 are located and cut at the end proximal to the spinal column, leaving the distal ends innervating the skin. This process is repeated on the opposite flank. The skin is then removed completely and transferred to the recording dish. Given the delicate nature of this surgical procedure it was not always possible to preserve all ten dorsal nerves.

Recording dish

We designed and built a novel recording dish (Figure 3-A) that allows for simultaneous epidermal stimulation and dorsal nerve recording. The skin-nerve prep is placed epidermal side down on the bottom of the dish, covering a hole just smaller than the section of skin. A rectangular cap fits over the edges of the skin, and screws apply pressure on all four corners of the cap. In this way the skin forms a seal along the bottom of the dish. The epidermal side of the skin remains accessible for stimulation. The inside of the skin, and attached dorsal cutaneous nerves, faces up, and the dish can be filled with recirculating, temperature-controlled, oxygenated (95/5 in a bicarb buffered solution) HEPES modified holding ACSF (NaCl: 92mM, KCI: 2.5mM, NaH_2PO_4: 1.2mM, $NaHCO_3$: 30mM, HEPES: 20mM, glucose: 25mM, sodium ascorbate: 5mM, thiouria: 2mM, sodium pyruvate: 3mM, $MgSO_4.7H_2O$: 2mM, $CaCl_2.2H_2O$: 2mM) to maintain viability of the nerves throughout the recording session (Figure3-B). The

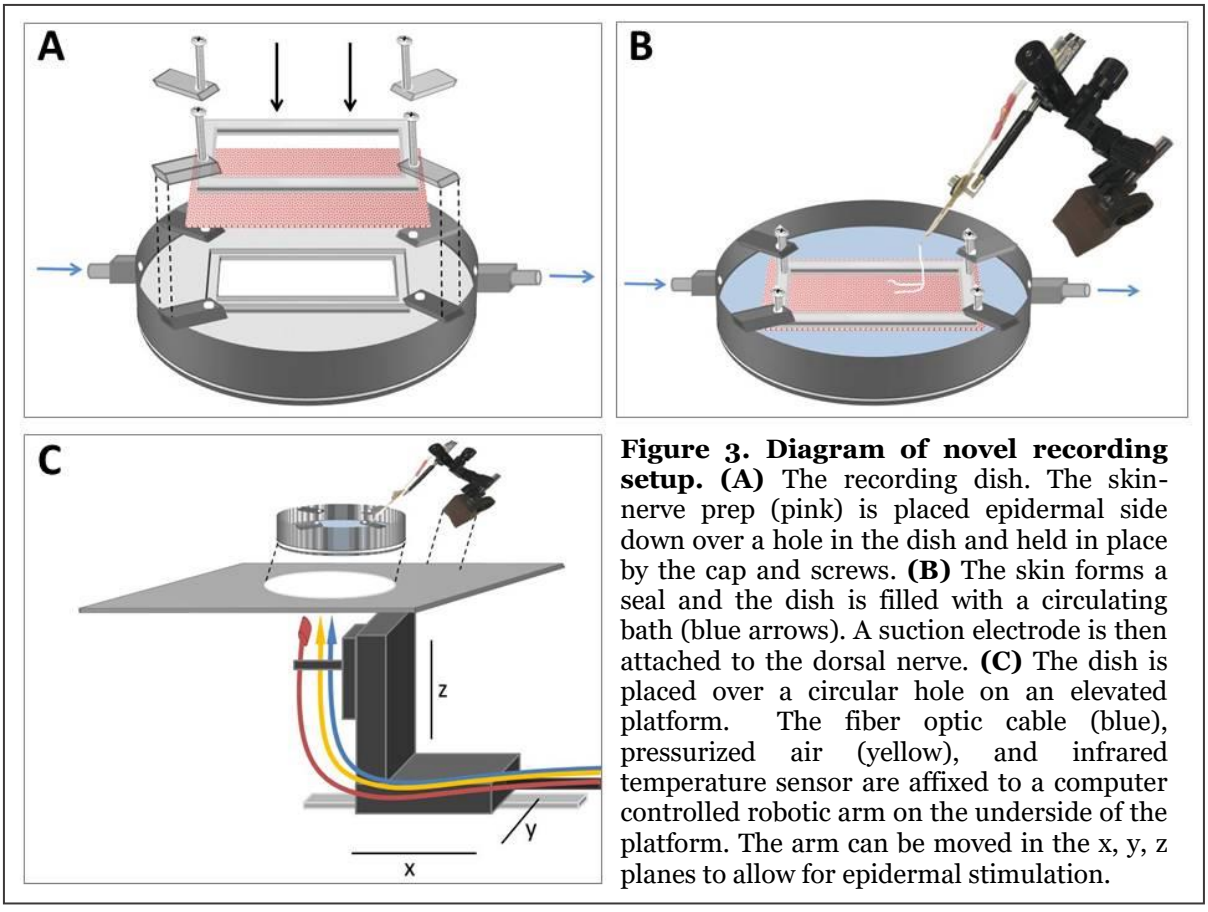


Figure 3. Diagram of novel recording setup. (A) The recording dish. The skin-nerve prep (pink) is placed epidermal side down over a hole in the dish and held in place by the cap and screws. **(B)** The skin forms a seal and the dish is filled with a circulating bath (blue arrows). A suction electrode is then attached to the dorsal nerve. **(C)** The dish is placed over a circular hole on an elevated platform. The fiber optic cable (blue), pressurized air (yellow), and infrared temperature sensor are affixed to a computer controlled robotic arm on the underside of the platform. The arm can be moved in the x, y, z planes to allow for epidermal stimulation.

perfusion rate was 70mL/min and monoamine neuromodulators were applied via pipette to an elevated chamber that supplied the bath with oxygenated, temperature-controlled solution through gravity-fed tubing attached to a reservoir. Solution leaving the recording chamber was recirculated back into the elevated reservoir chamber using a Masterflex easy-load II peristaltic pump (Cole-Parmer, Vernon Hills, IL). Drug washout was achieved by redirecting the solution exiting the bath into a disposal container and replacing the reservoir with a total of 300mL of new solution washed through the recording chamber prior to reinstating solution recirculation. An in-house made Peltier device and a Brushless DC fan (Orion Fans, Dallas, TX) were used to set and maintain temperature across an adjustable range. In these experiments, bath temperature was set at a physiologically appropriate value that we found to be most effective for optimal recruitment (26°C).

Recording setup

The recording dish sits over an opening on an elevated platform (Figure 3-C). A computer-controlled robotic arm is positioned beneath the opening, and is outfitted with a fiber optic cable for optogenetic stimulation, a tube for air pressure mechanical stimulation, and an infra-red temperature sensor to monitor changes in epidermal temperature. It is possible that either air puffs or laser stimulation could cool or heat the outside of the skin, respectively. It was important to monitor skin temperature, as most mechanoreceptors are responsive to temperature changes[1]. Observationally, there were no apparent changes in external skin temperature following either mechanical or optogenetic stimulation. The robotic arm is programmable to move in μm increments within an x, y, and z plane. This feature was developed for precise receptive field characterization, and to deploy mechanical stimulation while moving across the skin. It was not used for the purposes of this project. Glass suction electrodes were mounted on an electrode holder associated with a 3D micromanipulator that allowed for accurate mechanical positioning and flexible movement of tightfitting suction electrodes

enabling attachment to any of the ten dorsal nerves inside the recording dish (Figure 3-B). Evoked neural signals were amplified with an in-house built differential amplifier (2000x) and digitized at 50 kHz using a Digidata 1440A and Clampex software (Molecular Devices, San Jose, CA).

Optogenetic stimulation

We selectively activated TH^+ C-LTMRs using optical light pulses in our previously described lines of TH::ChR2-YFP mice. We used a 2W 445nm copper module laser with a 405-G-2 glass lens. A fiber optic cable delivered up to 4,846 mW/cm^2 of blue light to the epidermal side of the skin. Frequency, duration, and intensity of the light stimulus were controlled by the Molecular Devices computer program, Clampex.

Mechanical stimulation

Mechanical stimulation experiments were carried out in the same mice used for optogenetic experiments. We recruited various classes of mechanoreceptors using calibrated air pressure at forces ranging from 2 to 22 milliNewtons (mN). The precise amount of air flow delivered upon stimulation was monitored by a custom-built sensor, which output airflow measurements in volts. We conducted preliminary experiments using an in-house developed

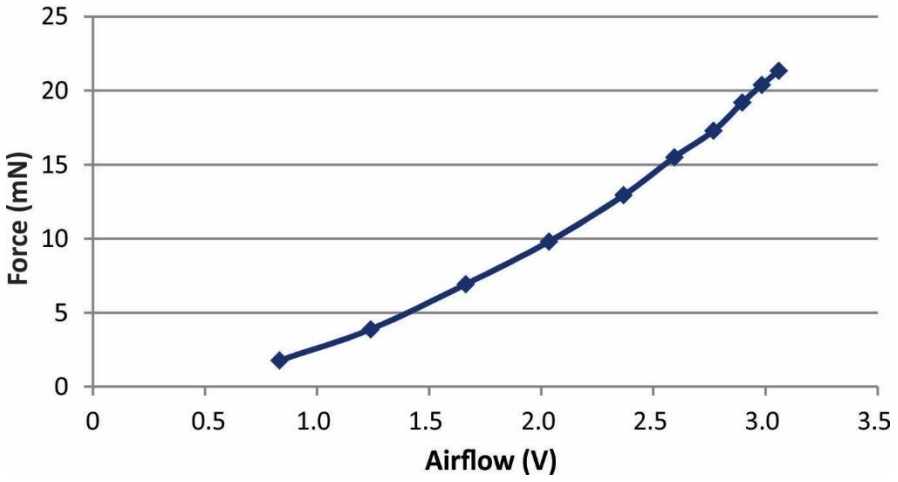


Figure 4. Ratio of airflow (in volts) as output by the computer program Clampex, to actual force (in mN) applied to the skin.

force plate to calibrate the actual amount of force delivered, in mN, compared to the computer readout, in volts (V), (Figure 4).

Experimental design

Optogenetic Recruitment of C-LTMRs

For this series of experiments, we utilized selective optogenetic recruitment of C-LTMRs in naïve (N=5), sham (N=2), and SCI (N=4) TH::ChR2-YFP mice. We assessed whether there are changes in the signaling properties of C-LTMR afferents in response to adrenergic, serotonergic, and cholinergic modulation. The experimental protocol is as follows. Suction electrodes were attached to one of five dorsal cutaneous nerves (T8 – T12). A computer-controlled robotic arm positioned the laser within the receptive field of the nerve on the hairy side of the skin. For recordings, I chose an area within the receptive field that elicited a maximal response. The recording program Clampex recorded activity from the nerve while delivering stimulation. Optogenetic recordings consisted of data collection files selected for episodic capture of 10 events ("sweeps") each consisting of a 500ms baseline recording (no stimulation), followed by 2000 ms of optogenetic pulses (10ms pulses at 5Hz frequency for a total of 10

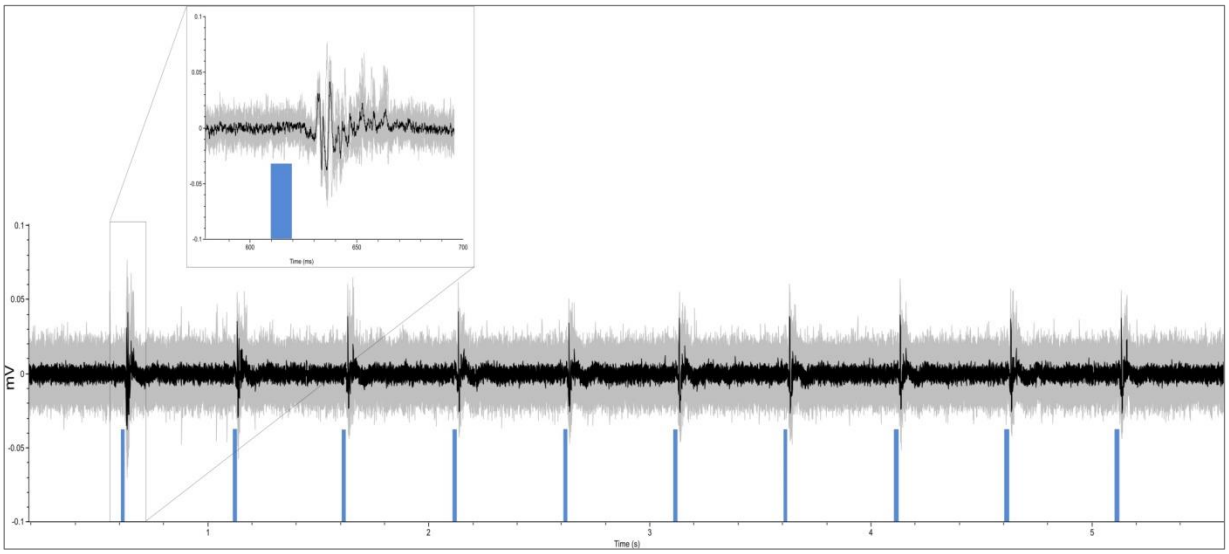


Figure 5. Example of an optogenetic stimulation recording. Blue lines indicate 10ms pulse of laser stimulation at 5 Hz frequency (10 pulses total). Grey signal is all ten sweeps overlaid, and black signal is an average of the ten sweeps. Insert shows the first light pulse and resulting recruitment. Recording included a 500 ms baseline and 2500 ms recovery period before and after stimulation, respectively (not shown).

pulses), and finally a 2500 ms long stimulation-free recovery period (Figure 5). For each experimental manipulation, three optogenetic recordings were collected in three consecutive files for a total of 30 episodic 5 Hz stimulation epochs (Figure 6-B). Prior to any drug administration, baseline recordings were obtained. During drug manipulations, a neuromodulator was added to the circulating bath and optogenetic stimulation recordings commenced immediately. After drug recordings the bath was replaced with new solution and "washout" recordings were obtained to assess recovery to baseline levels. We tested the effects of the adrenergic, serotonergic, and cholinergic neuromodulation. To assess adrenergic modulation we obtained recordings during each of the following drug manipulations: a norepinephrine transport inhibitor (NTI) clomipramine (5 μM), and norepinephrine at 1 μM, 10 μM, and 100 μM concentrations. Similarly, serotonergic modulation was assessed using the serotonin transport inhibitor (STI) citalopram (1 μM), and the same three increasing concentrations of serotonin. Cholinergic modulation was assessed using a selective nicotinic agonist, epibatidine (0.1 μM), the cholinesterase inhibitor neostigmine (20 μM), and acetylcholine at 10 μM concentration. A complete washout of the

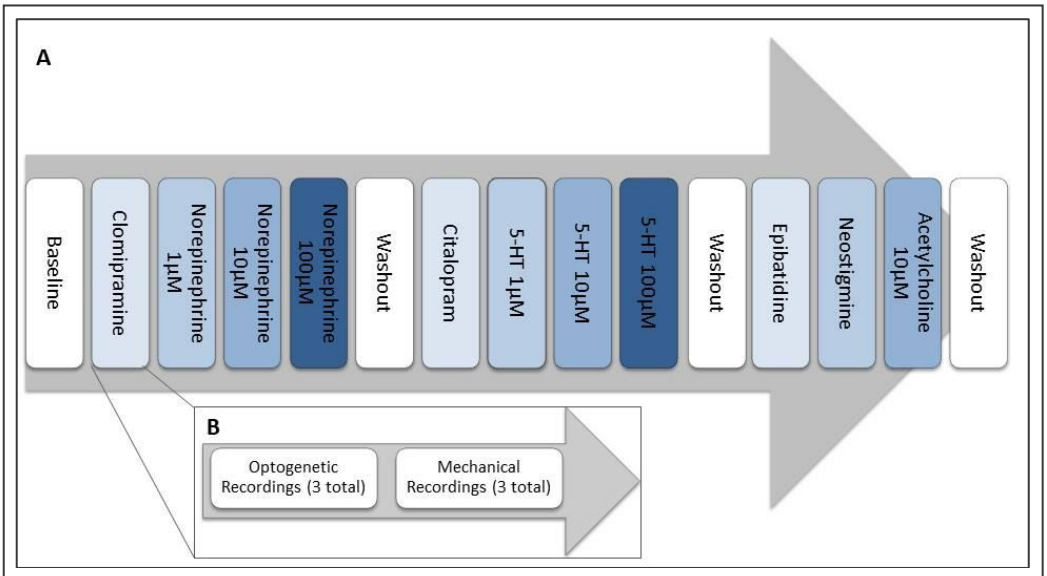


Figure 6. Schematic of the experimental design. (A) Timeline showing a representative order of neuromodulator group administration. The order of administration was randomized across animals. **(B)** For each segment, 3 consecutive files captured optically evoked events, followed by 3 consecutive files capturing air pressure evoked mechanical stimulation events.

drug was performed following the final cumulative dose, and preceding the next series of neuromodulator drug administrations (Figure 6-A). Drug administration order was randomized across animals. At the conclusion of the entire experiment the length of the nerve was measured for assessment of afferent axonal conduction velocity.

Mechanical Recruitment of Mechanoceptors

In addition to optogenetic stimulation we applied mechanical stimulation, in the form of airflow at calibrated forces, to analyze changes in the recruitment of the population of LTMRs recruited over a range of skin indentation forces. We assessed changes in mechanically evoked activity in response to the same monoamine neuromodulators as the optogenetic recruitment paradigm. For each baseline, drug administration, or washout condition, mechanical recordings were obtained directly following the optogenetic recordings. These consisted of episodic capture files composed of 10 sweeps each with a 500ms baseline, followed by 1000ms of continuous airflow and a final 2500 ms recovery period. Sweep 1 delivered air stimulation at a force of 2

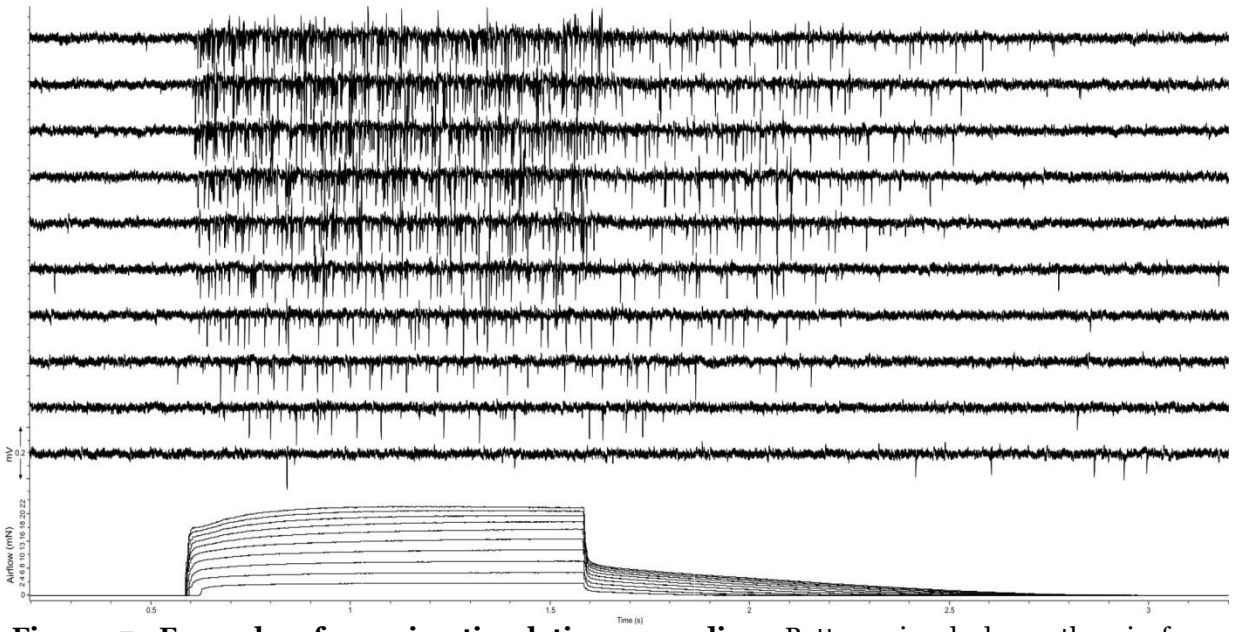


Figure 7. Example of an air stimulation recording. Bottom signal shows the air force increasing with each sweep. Upper signals; from bottom to top, sweep 1 through ten shows increasing activity with increases in air force. Sweeps delivered 2, 4, 6, 10, 13, 16, 18, & 22 mNs of force. Recording included a 500 ms baseline and 2500 ms recovery period before and after stimulation, respectively.

milineutons (mN), and subsequence sweeps delivered 4, 6, 10, 13, 16, 18, and 22 mNs (Figure 7). Three mechanical recordings were obtained for a total of 30 sweeps. The experimental protocol for mechanical stimulation, administration of neuromodulators, and nerve length measurement was the same as that described above.

Statistical Analysis

Optogenetic Recruitment of C-LTMRs

We assessed three attributes of our optogenetically recruited afferents: conduction velocity, overall afferent activity, and fatigability. Conduction velocity was measured as a function of nerve length and arrival time. We performed preliminary experiments to determine a more specific time-point of recruitment within the 10ms light pulse. Based on these experiments we used the 5ms time point to calculate arrival time for conduction velocity measurements. There were often multiple units recruited by a single light pulse; thus, CV is reported as an average of the first and last arriving events. Given the recruitment of multiple units, total C-LTMR recruitment was measured as the peak amplitude of the rectified integral (mv*s) of the signal containing all recruited events. A time-matched equivalent area of noise containing no events was rectified and integrating and its value was subtracted from the evoked value to produce a precise measurement (henceforth termed “response amplitude”) of overall C-LTMR recruitment per light pulse. Note that while such modification does not identify total number of units recruited, it allows comparison of relative changes in afferent recruitment. Sweeps containing spontaneous events (as determined by their unique waveform) that overlapped with the recruited events were removed from the analysis. To assess afferent fiber recruitment fatigability we compared the response magnitude of events recruited by the first light pulse, to those recruited by the last.

Recruitment & Fatigability of C-LTMRs in SCI versus Sham animals

To determine whether spinal cord injury alters recruitment of C-LTMRs we performed a student's t-test for independent variables on the response magnitude values of baseline recordings in SCI versus sham animals. Due to differences in the genetic lines, naïve animals were not used for comparison. To determine fatigability we used values representing the percent change in response magnitude from pulse 1 to pulse 10, and performed the same analysis as above. We did not assess differences based on the amount of time post-surgery because of the variability across animals.

Neuromodulation of C-LTMRs

Drug Order

Due to the nature of our experimental design, it was important to first determine if there was an effect of drug administration order (i.e. first, second, or last). To do so we used a multifactorial ANOVA on the raw response magnitude values. There was a significant order effect, despite the order randomization across animals. This may be due to the low number of animals used. Additionally, it is possible that there was simply a time-dependent reduction in recruitment caused by loss of suction quality or degradation of the skin-nerve preparation. In order to exclude loss of suction as a possible explanation I compared the amplitude of spontaneous individual axonal events that were present for the extent of each experiment. There was no significant difference in response magnitude between spontaneous events quantified from the start compared to those seen at the end of the experiment (Figure 8), suggesting that reduced suction quality cannot account for these differences. I cannot exclude the possibility of reduced recruitment of individual afferents due to time-dependent changes in physiological status of the prep as there was clearly still a time-related issue in evoked amplitude. Alternately, this reduction could be explained by a lack of recovery after washout. We

were unable to discriminate between these two possibilities. In order to mitigate this time-dependent reduction in evoked responses, and to ensure that our analysis was strictly of drug-dependent changes, response magnitude values for each drug were normalized to their preceding washout value (termed "pre-wash"). This value was normalized to 100%, with subsequent drug administration and post-drug washout (termed "post-wash") reported as a percentage of the pre-wash.

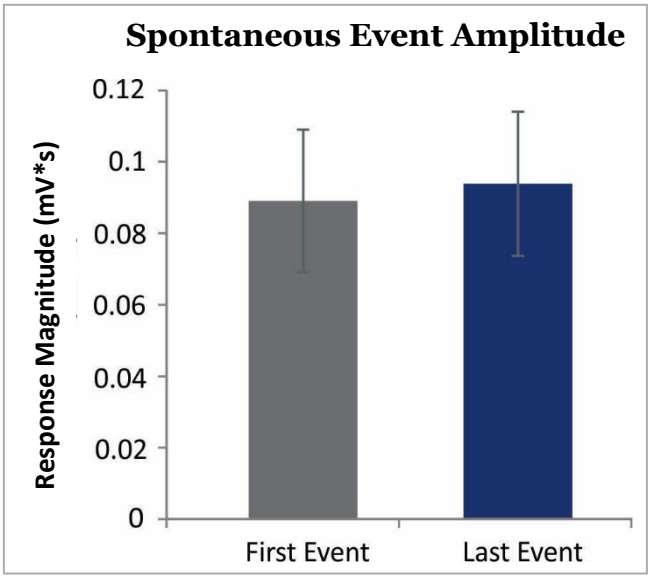


Figure 8. Response magnitude of spontaneous events at the beginning and end of each experiment. There was no significant difference between the first and last events (N=10, P=0.87). This confirms that changes in amplitude of evoked events over the course of the experiment are not due to degradation of suction quality or long-term viability of the skin-nerve preparation.

SCI versus Control

To assess differences between SCI and control groups with regard to a drug effect we used a multifactorial ANOVA. We did not find a significant drug X group effect; therefore, we performed the rest of the drug analyses with all groups binned.

Adrenergic, Serotonergic, & Cholinergic Modulation

Using the normalized values we performed a one-way ANOVA for each drug group independently. A Tukey's post-hoc was used to assess specific changes between each condition: pre-drug baseline, reuptake/cholinesterase inhibitor, each drug concentration, and post-drug wash.

Mechanical Recruitment of Mechanoceptors

Consistent with the optogenetically recruited events, we used response magnitude as a measure of total mechanoceptor recruitment during air stimulation. The one second period of

air stimulation contained a great number of events. In some instances, it was clear that unit firing continued past the period of stimulation. However, this effect was not consistent across animals or even recordings. To maintain consistency in our response magnitude measurements we opted to only rectify and integrate the signal pertaining to the period of stimulation. Additionally, it is important to note that, unlike the optogenetic stimulation recordings, it was often impossible to differentiate between evoked and spontaneous events arriving within that window of stimulation. Therefore, analysis was performed on all sweeps, regardless of potential spontaneous activity. It is quite possible that the monoamine neuromodulators we used could have effects on spontaneous firing. It is unlikely that the occurrence of spontaneous activity, or changes thereof following drug administration, is significant enough to impact our results, particularly because to obtain our measurement of evoked response we subtract an equivalent area of noise. To assess baseline differences in recruitment at each force we ran a one-way ANOVA with Tukey's post-hoc comparing the following forces to one another: 2, 4, 6, 10, 13, 16, 18, & 22mN. Next, we tested the effect of each drug per force, again using a one-way ANOVA with Tukey's post-hoc. For the mechanical recruitment experiments we did not assess differences between SCI and control.

Results

Assessment of changes in optogenetically recruited C-LTMRs

Evidence that the optogenetic approach selectively recruited C-LTMRs

Optogenetic recruitment of TH+ afferents in the skin was used to assess the activity of C-LTMRs at baseline and following neuromodulation in naïve, sham, and SCI animals. Using two TH-Cre-ER lines (JAX # 008532 JAX # 025614) crossed with ChR2-eYFP mice (JAX # 012569) we were able to successfully use fiber-optic light pulses to recruit afferents in the skin. This recruited population has an average conduction velocity (CV) of 0.56 m/s, which falls within the

literature-reported range for C-LTMRs (0.4 – 2 m/s)[14, 20]. As we recruited many afferents and they fatigued over time we chose not to study variability in conduction velocity but rather just overall changes in amplitude. C-LTMRs are known to be highly fatigable[16]. Consistent with this, the ability to optogenetically recruited afferents was dramatically reduced by 72% by the 10th pulse of a 5 Hz train compared to its initial value (Figure 9).

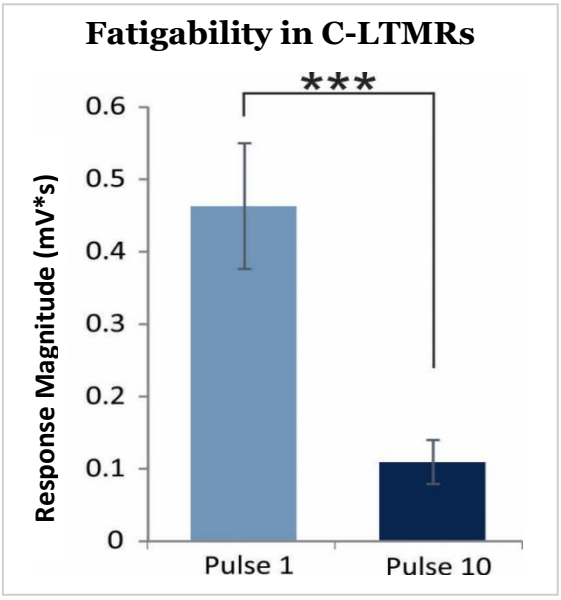


Figure 9. Optogenetically recruited C-LTMRs were highly fatigable at baseline. Recruitment of C-LTMRs was dramatically reduced from pulse 1 to pulse 10 (P=0.001). Error bars presented as SEM, N=10.

SCI leads to a reduction in recruitment of C-LTMRs

Since the naïve animals in this study were from a different genetic line than the sham and SCI groups, they could not be used as a comparison group in our analysis of SCI - induced changes in C-LTMR recruitment. Therefore, we compared the response magnitude of optogenetically recruited C-LTMRs between the sham and SCI groups only. It should be noted that this left us with a small number per group. Even so, the amplitude of recruited events was significantly higher in the sham animals (Figure 10). These results should be interpreted with caution given the low number of animals; however, they do suggest that SCI reduces C-LTMR recruitment. Additionally, recordings in SCI animals were obtained from dorsal nerves T10 (N=2) T11, and T12 (N=1 each) so there could be differences in recruitment levels based on proximity to the level of injury (T10), namely that injury axotomized the central axons, leading to a reduced response magnitude at the T10 dorsal root. Nonetheless, it appears that in some way SCI is altering the activity of C-LTMRs.

SCI does not appear to alter fatigability in C-LTMRs

Similarly to C-LTMR recruitment, we tested the effect of SCI on a key property of these afferents: fatigability. Our analysis did not find a significant difference in fatigue levels between the SCI and sham groups (Figure 11). However, this analysis was under-powered given the low number of animals. As such, there may indeed be an effect of SCI on fatigue in C-LTMRs that we were simply unable to detect. Further studies will need to be conducted to assess injury-induced changes in fatigability.

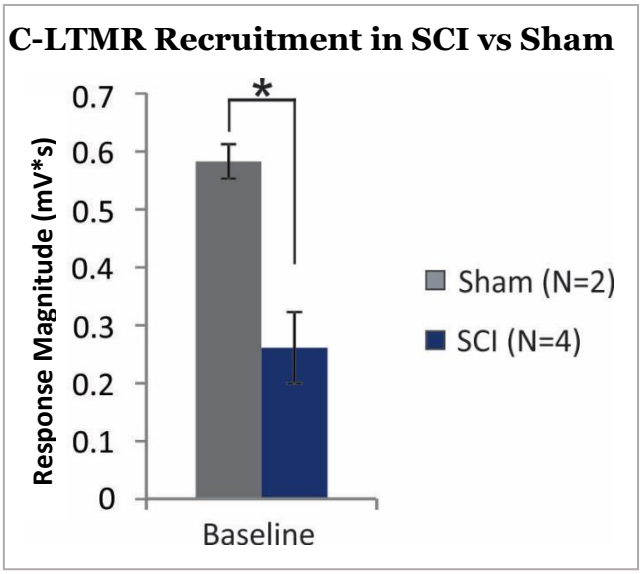


Figure 10. Recruitment of optogenetically evoked C-LTMRs at baseline. C-LTMR recruitment in the sham group was significantly higher than in the SCI group (P=0.04). Error bars are presented as SEM.

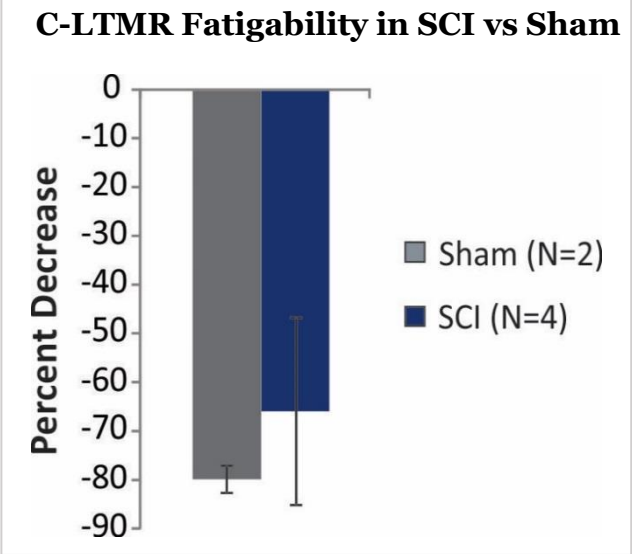


Figure 11. Fatigability of optogenetically evoked C-LTMRs at baseline. There was no significant difference in fatigability between the sham and SCI groups (P=0.7). Values shown as percent decrease in response magnitude from pulse 1 to pulse 10. Error bars are presented at

Neuromodulation-induced changes in C-LTMRs

Adrenergic modulation reduces C-LTMR recruitment

When compared to the immediately preceding washout condition, the norepinephrine transport inhibitor (NTI) clomipramine (5µM concentration) significantly reduced the response magnitude of recruited events by 35.4% (Mean percentage of pre wash=64.59± 23.34, P=0.02)

(Figure 12). This suggests that there is an endogenously available source of norepinephrine in the skin milieu, and blocking its reuptake can result in decreased C-LTMR activity. Subsequent direct application of norepinephrine at progressively increasing concentrations 1, 10, and 100 μM did not lead to any additional changes in response strength, suggesting that transport block increases in endogenous levels of bioavailable noradrenaline whose depressive action is already maximal. There were no significant differences between the modulatory conditions, indicating that increasing amounts of norepinephrine do not result in a compounding reduction in C-LTMR activity. There was not a significant difference between the pre-wash and post-wash conditions; however, there was also not a significant difference between clomipramine or any of the norepinephrine concentrations and post-wash. Together these results indicate that wash led to a partial recovery of C-LTMR activity levels. As adrenergic applications were never undertaken prior to application of citalopram, we presume but do not know whether actions would be evoked in the absence of transport block.

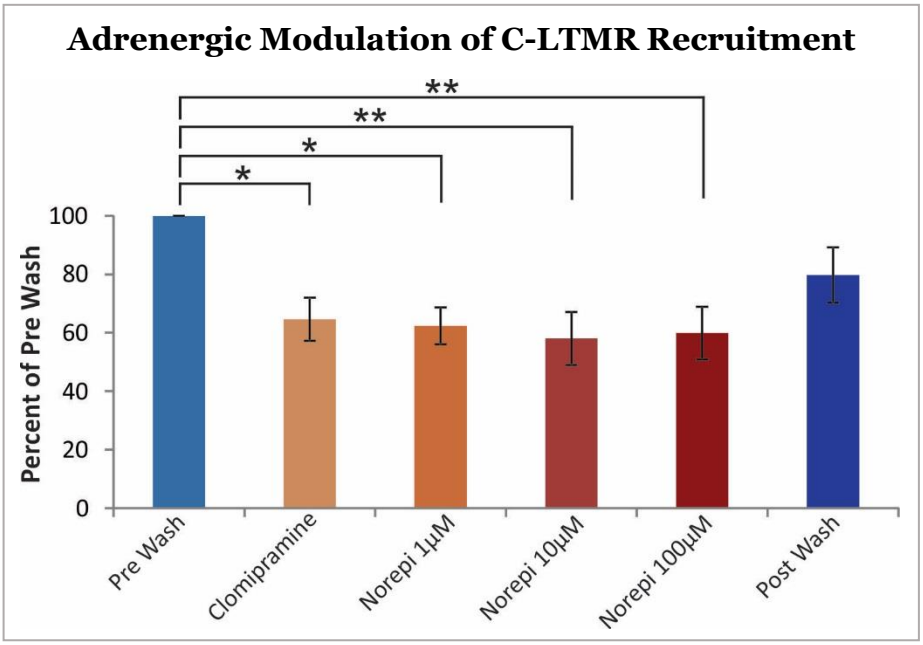


Figure 12. Effects of an NTI and varying doses of Norepinephrine during optogenetic recruitment of C-LTMRs. C-LTMR recruitment was significantly reduced during administration of the NTI clomipramine (P=0.02), and all three concentrations of norepinephrine: 1μM (P=0.01), 10μM (P=0.003), and 100μM (P=0.005) as compared to the preceding wash. There were no significant differences between each drug administration. Error bars are shown as SEM, N=10.

Adrenergic modulation does not alter C-LTMR fatigability

Adrenergic modulation did not significantly change C-LTMR fatigability as compared to baseline. (Values reported as an average percent decrease in response magnitude from pulse 1 to pulse 10: Baseline=80.4 ± 16.4, Drug=72.1 ± 28.8, P=0.8)

Serotonergic modulation reduces C-LTMR recruitment

Serotonergic modulation also reduced optogenetic recruitment of C-LTMRs (Figure 13). Bath administration of the serotonin transport inhibitor (STI) citalopram resulted in reduced levels of activity (Percent of pre-wash average=69.04 ± 19.06, P= <0.01). Similarly to the NTI, this result indicates the endogenous release of serotonin in the skin, as well as its ability to modulate C-LTMR function. Though there was a trend toward further dose-dependent reduction in the evoked response following application of serotonin (1, 10, & 100 μM), the responses were not significantly different than those observed with citalopram. Our small animal numbers did not provide the desired power level that could support a relationship between serotonin levels and extent of C-LTMR silencing. For example it looks like a further 20% decrease in response was seen after citalopram when the higher doses of serotonin were applied leading to highly significant reductions compared to prewash baseline. Further studies

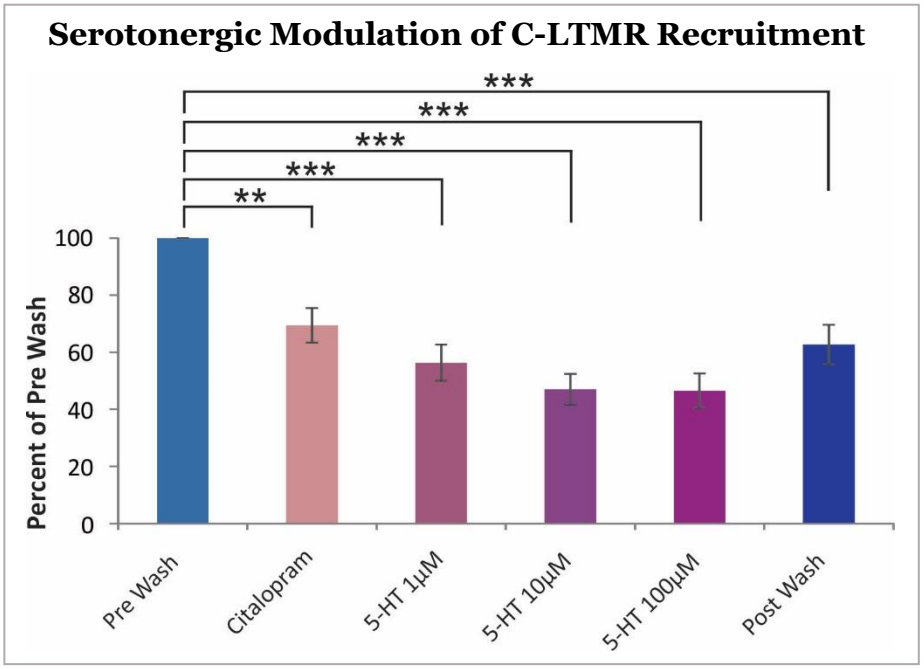


Figure 13. Effects of an STI and varying doses of serotonin during optogenetic recruitment of C-LTMRs. C-LTMR activity was significantly reduced during administration of the STI citalopram (P=0.007), and all three concentrations of serotonin: 1μM (P=0.0001), 10μM (P=.0001), and 100μM (P=0.0006) as compared to the preceding wash. There were no significant differences between each drug administration. Error bars are shown as SEM, N=10.

are needed to determine if this relationship exists. Finally, there was a significant decrease in response magnitude from the pre-drug baseline compared to post-drug washout. Thus, washout was not effective at reversing the modulatory effect of serotonin. This could be due to two things: the washout did not successfully remove serotonin from the synapses, or serotonin modulation has long-lasting effects on C-LTMR activity.

Serotonergic modulation does not alter C-LTMR fatigability

There were no serotoninergic modulatory actions on levels of fatigue in optogenetically recruited C-LTMRs as compared to baseline (Values reported as an average percent decrease in response magnitude from pulse 1 to pulse 10: Baseline=76 ± 25.4, Drug =83.6 ± 28.8, P=0.85).

Cholinergic modulation does not significantly affect C-LTMR recruitment

Unlike noradrenaline and serotonin, cholinergic modulation did not significantly decrease the optogenetic recruitment of C-LTMRs (Figure 14). Administration of the selective nicotonic receptor agonist epibatidine did not have a significant effect on C-LTMR activity; however, it did appear to trend towards a decrease in recruitment (N=6, Mean=62.52 ± 17.68, P=0.26). Only six of the animals received epibatidine and as such the analysis was under powered. Similarly, the

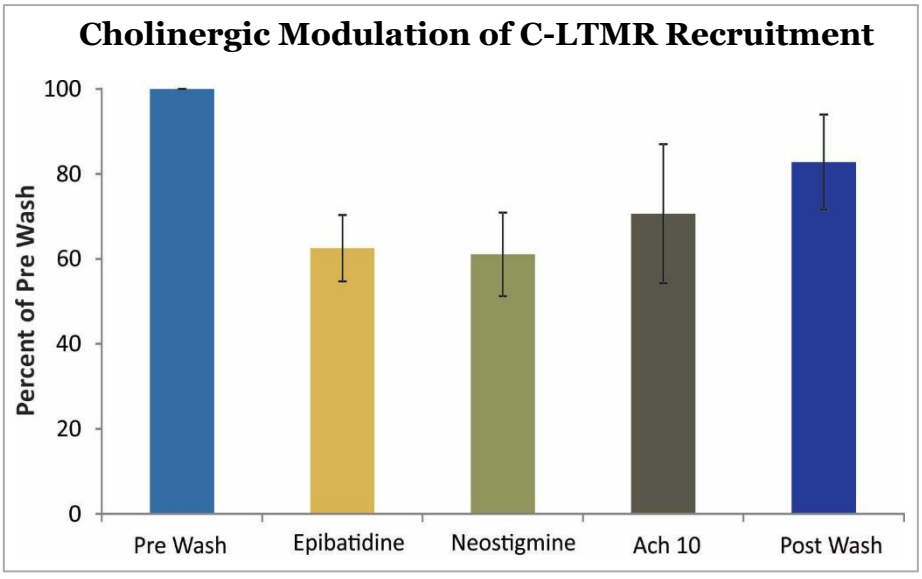


Figure 14. Effects of a selective nicotinic agonist, cholinesterase inhibitor, and 10μM acetylcholine during optogenetic recruitment of C-LTMRs. C-LTMR recruitment is not significantly decreased during cholinergic drug administration. Error bars are presented as SEM. Epibatidine N=6, Neostigmine, Ach 10, and Wash

cholinesterase inhibitor neostigmine did not cause significant reduction (P=0.1), although it did appear to trend in that direction. Direct application of acetylcholine (10 μM concentration) also did not have a significant effect on activity levels. It is important to note that these analyses are confounded due to the different experimental protocols used in the naïve versus sham/SCI groups. Specifically, the naïve groups did not receive epibatidine prior to neostigmine administration. Therefore, we performed a second analysis with the two groups (epibatidine+neostigmine vs neostigmine alone) separately. Interestingly, we found that in the absence of epibatidine, neostigmine significantly reduced the response magnitude of C-LTMRs (N=6, Mean=52.16 +/- 22, P=0.04) (Figure 15). While there was a trend in reduction in the group that received both epibatidine and neostigmine, the results were not significant (N=6, Mean=67.02 +/- 36, P=0.26). These findings could indicate an interaction between epibatidine and neostigmine, but it is more likely that the epibatidine group simply had a higher amount of variability. Further exploration into the effects of cholinergic modulation on C-LTMR activity is needed.

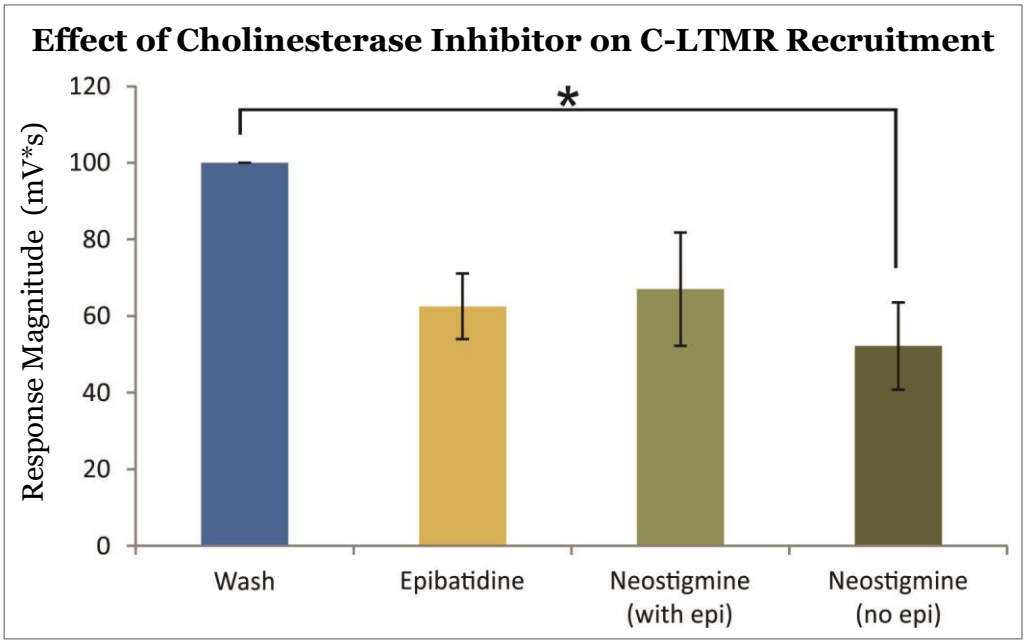


Figure 15. Effect of neostigmine on C-LTMR recruitment in the presence or absence of epibatidine. When neostigmine was applied following epibatidine it did not result in a significant reduction in response magnitude (N=6, P=0.26). However in the animals that did not receive epibatidine, neostigmine did significantly reduce the response magnitude of C-LTMRs (N=4, P=0.04).

Cholinergic modulation does not alter C-LTMR fatigability

C-LTMR fatigability was not altered following cholinergic modulation as compared to baseline (Values reported as an average percent decrease in response magnitude from pulse 1 to pulse 10: Baseline=72.5 ± 27.4, Drug average=72.3 ± 24.9, P=0.74).

Mechanical recruitment of Low Threshold Mechanoreceptors

Mechanical stimulation, in the form of air pressure pulses calibrated across a range of forces, was used to assess monoamine neuromodulation on summed collective classes of recruited mechanoreceptors. Varying forces should, in theory, recruit different classes of afferents (See table 1). Therefore, based on the force we can broadly assess the modulatory effects of norepinephrine, serotonin, and acetylcholine on different classes of mechanoreceptors. Increases in force corresponded with higher values of summed response magnitude (Figure 16). This relationship was maintained throughout each washout (Figure 17) as well as all drug applications. It is noteworthy that response magnitude undergoes its greatest reduction between baseline control conditions and first washout of drug (Wash 1) after which baseline stabilizes. Whether this initial stabilization is due to the first series of drug applications or a time-dependent feature of the system cannot be determined from these experiments. As progressively larger forces would be expected to recruit additional afferent LTMRs with higher force thresholds, as well as increased firing frequency in already recruited populations increases in response magnitude should be interpreted as the summed physiologically encoded sensory barrage. In this way changes in overall strength of sensory response by neuromodulators represent changes in peripheral somatosensation.

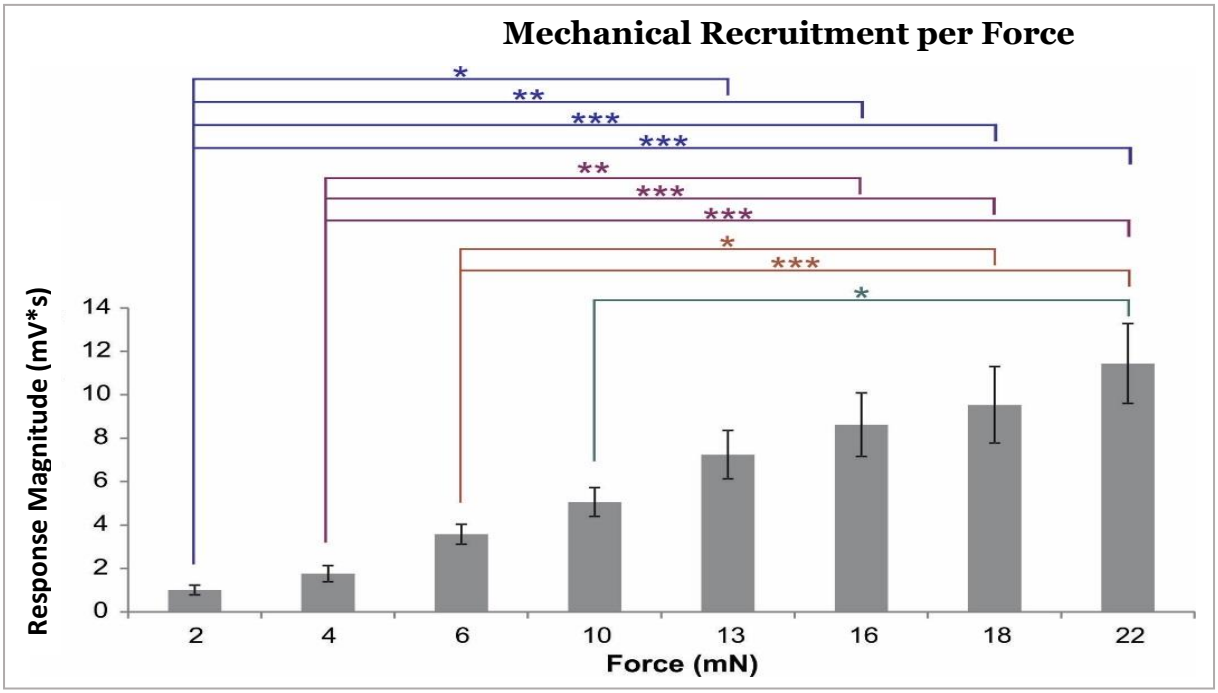


Figure 16. Response magnitude of mechanically recruited afferents increases per force. Recruitment values of each force were significantly different from those of the force four steps above or higher. For instance, activity recruited at 2mN was only significantly different than that recruited at 13 mN or higher. Error bars represent SEM, N=8.

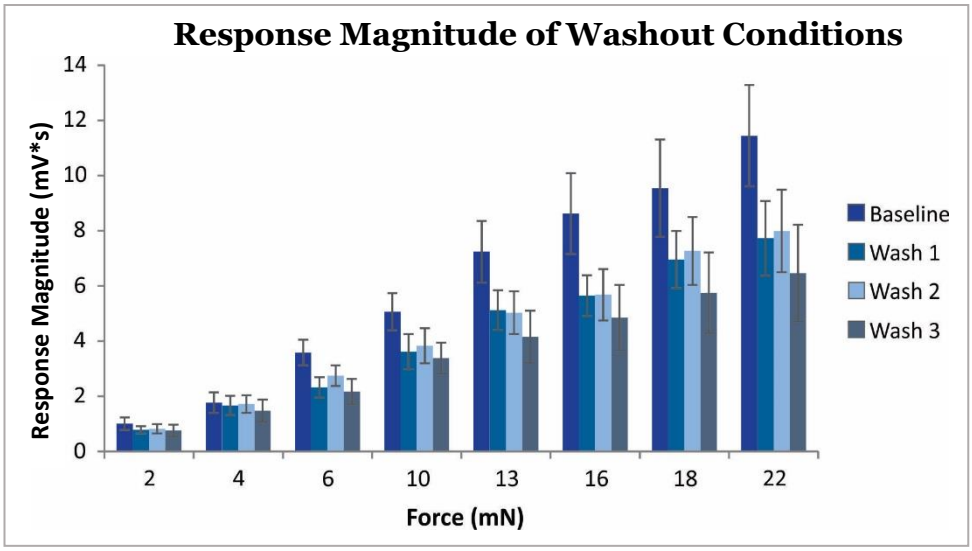


Figure 17. Relationship between force and response magnitude. Recruitment decreases as a function of time, but the relationship between force and response magnitude is maintained. Error bars represent SEM, N=8.

Adrenergic Modulation of LTMRs

Activity of LTMRs at each force was reduced in response to adrenergic modulation (Figure 18). Specifically, the NTI clomipramine reduced activity at 2 and 13 mN forces, demonstrating that endogenously released norepinephrine can depress LTMR activity. There was a trend of reduction at all other forces, but it did not reach significance. At a concentration of 1μM, direct application of norepinephrine resulted in significantly reduced activity at 2, 13, 16, and 18mN forces. Norepinephrine at 10 μM concentration only lowered activity at the higher forces (10, 13, 16, 18, and 22mN). At the highest concentration, norepinephrine reduced afferent activity at all forces. These results indicate that (a) blocking the reuptake of norepinephrine can result in endogenous adrenergic modulatory depression of LTMRs, (b) direct application of norepinephrine in the presence of a reuptake inhibitor further reduces response magnitude, and (c) magnitude of response depression is broadly concentration dependent: the highest dose leads to the largest overall depression. However these differences are not substantial and overall

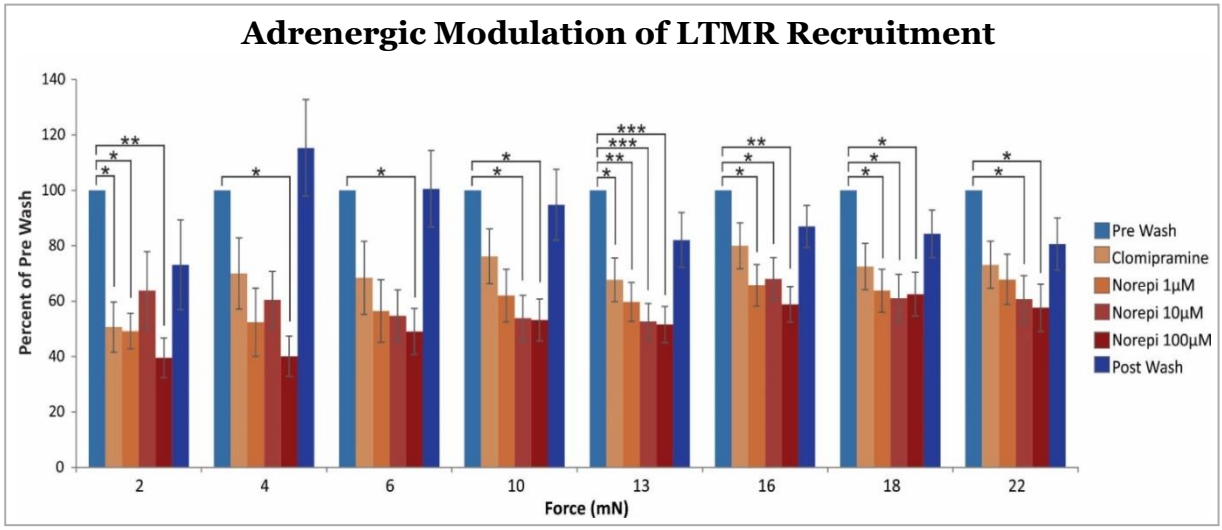


Figure 18. Effects of an NTI and varying doses of Norepinephrine during mechanical stimulation. Overall recruitment of mechanoceptors decreases when drug is present. The highest concentration of norepinephrine (100μM) significantly decreased recruitment at all forces, while the NTI and two lower concentrations (1 & 10 μM) had varying results depending on force. Values represented as a percentage of the preceding washout condition. Error bars are presented as SEM, N=8)

depression after subsequent application of norepinephrine was not statistically greater than that seen with clomipramine. This dose curve could be attributed to several factors: low concentrations only affect certain classes of afferents while higher concentrations have more broad-reaching effects, or increasing the concentration leads to suppression of a higher number of individual units.

Serotonergic Modulation

Serotonergic modulation reduced LTMR recruitment at all forces except 6mN (Figure 19). Specifically, the serotonin transport inhibitor (STI), citalopram, significantly reduced recruitment of units at forces 2, 4, and 13mN. These results indicate that serotonin is endogenously synthesized, released and available in the skin milieu for modulatory depression of LTMRs. Like norepinephrine, trends toward depression were observed throughout the range of force perturbations assessed, yet there is preferential depression of afferents recruited at the lowest at midrange forces. Exogenously added serotonin also appears to contribute to reduced activity. At a concentration of 1 µM it significantly decreased activity of afferents recruited by 2mN of force, as well as those recruited by 13 and 18 mN. Increasing the concentration to 10 µM

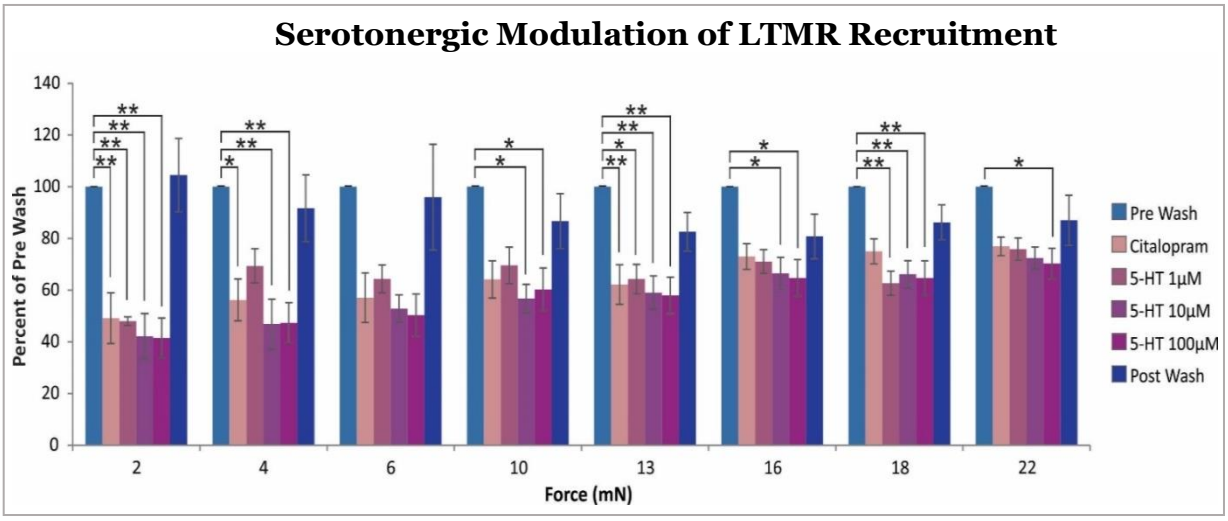


Figure 19. Effects of an STI and varying doses of Serotonin during mechanical stimulation. Overall recruitment of mechanoceptors decreases when drug is present at most, but not all forces. Values represented as a percentage of the preceding washout condition. Error bars are presented as SEM, N=8)

resulted in more broad-reaching suppression at forces 2, 4, 10, 13, 16, and 18 mN. The final concentration of serotonin (100 μM) maintained the effects seen at lower concentrations, and further resulted in reduced activity of afferents recruited at 22 mN. Overall however, given that serotonin did not lead to significantly greater depression compared to citalopram it is clear that the bulk of the depressive response is already produced following block of endogenous uptake. One notable observation is that serotonergic modulatory actions appear to preferentially recruit the lowest threshold afferents: the greatest depression was seen at the lowest force tested and the least depression was observed at the highest force tested (although we did not test the significance of this relationship). Overall these results suggest that LTMRs are amenable to endogenous depressant neuromodulation by serotonin and that these actions are preferential to afferents recruited at the lower force ranges.

Cholinergic Modulation

Cholinergic modulation of LTMR activity was restricted to direct application of acetylcholine (Figure 20). The selective nicotinic agonist epibatidine did not significantly alter overall activity of mechanically recruited afferents at any force. Similarly, the cholinesterase inhibitor neostigmine did not significantly change mechanically recruited activity. Application of acetylcholine (10μM) reduced the activity of LTMRs recruited at the lower force (4mN) and at the three highest forces (16, 18, 22mN), but not those recruited by intermediate forces. The actions of acetylcholine likely include actions on muscarinic receptors given that the observed depression was always numerically greater than epibatidine. Given the small sample size for epibatidine, it would be premature to exclude lack of observed significant reduction with epibatidine as demonstrating that observed actions with acetylcholine being exclusively via muscarinic receptors. Similarly, even though block of acetylcholine degradation with neostigmine did not lead to significant reductions in response magnitude, larger sample sizes

are required before one could conclude that there is an absence of endogenous cholinergic modulation of LTMR afferent input.

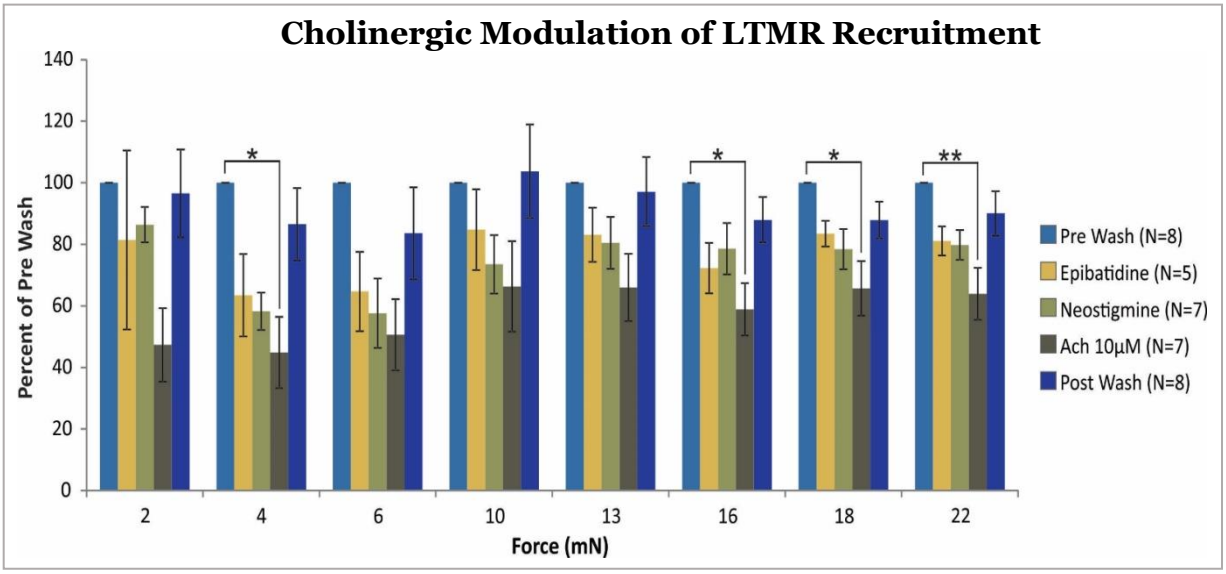


Figure 20. Effects of a selective nicotinic agonist, cholinesterase inhibitor, and 10μM acetylcholine during mechanical stimulation. Overall recruitment of mechanoceptors decreased only during application of acetylcholine, and not in response to the nicotinic agonist or cholinesterase inhibitor. This effect was only present at low recruitment forces (4mN, P=0.03) and high recruitment forces (16 [P=0.01], 18 [P=0.01], & 20mN [P=0.07]). Values represented as a percentage of the preceding washout condition. Error bars are presented as SEM.

Discussion

Low threshold mechanoreceptors play a vital role in interactions with our physical environment. They convey information regarding soft, gentle touch, movement across the skin, and hair follicle deflection, among other things[6]. Perception of these types of stimuli can become disrupted in neuropathic pain states after spinal cord injury, particularly in allodynia, which is characterized by painful sensations in response to innocuous tactile stimuli[94]. While central sensitization is known to play a role, alterations in peripheral sensory processing may also contribute to maladaptive pain syndromes following injury[39]. In particular, recent evidence has implicated a class of low threshold mechanoreceptors, C-LTMRs, which normally

encode for pleasant touch, in the development and maintenance of allodynia[18, 20, 84, 87, 88]. Several factors may contribute to altered peripheral processing, including inflammation, sympathetic activity, and neuromodulation. This project set out to explore the effects of three types of neuromodulation, cholinergic, adrenergic, and serotonergic, on peripheral sensory processing. Specifically, we looked at how such modulation might alter signaling in hairy-skin low threshold mechanoreceptors (LTMRs), with particular emphasis on C-LTMRs. Additionally, we examined the baseline response magnitude of C-LTMRs in spinal cord injured versus sham-injured animals. The following sections will discuss (a) the electrophysiological recording setup that we designed and developed, (b) the effect of SCI on C-LTMR response magnitude and fatigability, (c) the effects of norepinephrine, serotonin, and acetylcholine on C-LTMR response magnitude, (d) the effects of those same neuromodulators on hairy-skin LTMRs, (e) potential limitations of this study, and (f) future directions.

Novel Recording setup and genetic lines

In order to explore the relationship between neuromodulation and afferent activity, it was first necessary to develop a system that would allow us to access the outside of the skin for physiologically relevant stimulation, as well as access the inside for drug application and recording. Our first step was to develop a surgical procedure to detach a large section of hairy skin with intact dorsal cutaneous nerves. This skin-nerve preparation thus contains the outside of the skin for stimulation, and preserves the afferent innervation of hair follicles, while the proximal end of the nerve is exposed to allow for suction-electrode recording. Next, we needed a setup to hold the detached skin-nerve prep that would leave the outside skin accessible for stimulation, but submerges the inside in solution. This solution serves a three-fold purpose: it provides oxygenation to maintain the viability of the preparation, it serves as a medium to disperse drugs, and it allows for suction electrode recording from the nerves. A setup such as this did not exist, so we designed and developed a dish that used the skin itself to seal a hole in

the bottom of the dish. In this way the underside or outside of the skin was still accessible, and the inside of the dish could be filled with a circulating bath. Taken together, this preparation and recording setup can be adapted and utilized to study any number of peripheral manipulations and their impact on afferent fibers. For our experiments we outfitted the recording setup with air puff and optogenetic stimulation. The air puffs could be calibrated to deliver precise amounts of force, thereby recruiting a range of low threshold mechanoreceptors. We utilized optogenetic stimulation to selectively recruit C-LTMRs, which are of particular interest given their purported role in tactile allodynia[18, 20, 84, 88]. Tyrosine hydroxylase (TH) is a known enzyme in catecholamine biosynthesis and is selectively expressed in a large proportion of C-LTMRs[17]. However, it is also temporarily widely expressed in other afferents during development[95]. Therefore, TH-Cre genetic lines which have constitutively active cre-mediated expression will not selectively target C-LTMRs. Therefore, we utilized a genetic lines in which cre-mediated expression is only activated following administration of tamoxifen[95]. This approach not only ensures selective expression in C-LTMRs. To confirm expression we first performed immunohistological processing for TdTomato in the TH::TdTomato transgenic mouse to confirm selective expression in C-LTMRs at longitudinal lanceolate endings around hair follicles. Using our recording setup we confirmed that fiber optic stimulation of C-LTMRs in the skin of TH::CHR2 transgenic mice resulted in afferent firing characteristics consistent with C-LTMR properties. This genetic approach allowed us to analyze specific characteristics of C-LTMRs, but it can be adapted to target any afferent fiber.

Effect of Spinal cord injury on C-LTMRs

Spinal cord injury disrupts a great deal of normal function both centrally and in the periphery. Among these are perturbations in sympathetic function and chronic pain syndromes. Neuropathic pain is one of the most common SCI-induced pain syndromes, with nearly 50% of patients developing some form of it after injury[25]. Allodynia, which is characterized by a

burning, painful sensation in response to typically innocuous stimuli such as soft stroking of the skin[26, 94], is perhaps one of the most challenging forms of neuropathic pain as it can be triggered by the movement of clothes against the skin, or the gentle touch of a loved one. Historically, the mechanisms underlying allodynia were thought to involve altered signaling in the spinal cord[96] as a result of central sensitization wherein Aβ afferents project to dorsal horn nociceptive neurons[97, 98]. However, this hypothesis was developed before C-LTMRs were discovered in humans. The discovery of CT afferents opened the door for exploration into their role in tactile allodynia. Indeed, several studies have now established a clear link between C-LTMRs and the development and maintenance of allodynia[18, 19, 84, 85, 88]. While effective in determining the involvement of C-LTMRs in allodynia, these studies did not use SCI models of allodynia, therefore the question of the impact that SCI has on C-LTMR function remains unanswered. Our studies on the effects of SCI were done in collaboration with the Garraway lab. Prior to our *ex vivo* electrophysiological experiments they tested the SCI and sham animals for the development of mechanical hypersensitivity. Furthermore, using optogenetic stimulation on the trunk skin of awake, behaving animals and a conditioned place preference paradigm, they showed that the SCI animals developed an aversion to the chamber in which they were stimulated, while sham animals did not[99]. These preliminary results demonstrate that selective recruitment of C-LTMRs can induce a behavioral change indicating that the animals find the stimulation aversive. At the conclusion of these behavioral studies, we used the same animals for our electrophysiology experiments. We observed that optogenetic recruitment of these afferents was significantly diminished in the SCI group as compared to shams. We did not find an effect of SCI on C-LTMR fatigability. To our knowledge this is the first evidence that SCI can lead to altered peripheral signaling in C-LTMRs. Granted, these results were observed in a very small number of animals, so further experiments should be conducted to confirm our findings. Nevertheless, a reduction in C-LTMR recruitment after SCI would support the theory that allodynia is due to a loss of the normally pain inhibiting role of C-LTMRs[20]. Additionally,

we did not find any significant interactions between SCI or the three neuromodulators. It should be noted however that these analyses were underpowered so we cannot ignore the possibility that an effect exists but was undetectable.

Neuromodulation of C-LTMRs

There is ample evidence to suggest that C-LTMRs are necessary for the development and maintenance of tactile allodynia after injury. However the exact mechanisms remain unclear. Several studies have indicated that peripheral signaling may play a role in maladaptive processes after injury[38-40]. One potential means of altered peripheral signaling is neuromodulation. In the following sections we will discuss three neuromodulators, norepinephrine, serotonin, and acetylcholine, and their effects on C-LTMR recruitment.

Norepinephrine

Adrenergic innervation in hairy skin comes from a multitude of sources including adrenal glands, sympathetic free nerve endings, keratinocytes, and melanocytes[63, 65, 71-74]. The most likely source of norepinephrine that could interact with C-LTMRs comes from sympathetic fibers, which have been shown to form close physical associations with hair follicles afferents[45]. Furthermore, studies have demonstrated that C-LTMRs have increased activity following sympathetic stimulation, and changes due to blood flow or temperature were ruled out[46, 47]. This finding of increased activity following sympathetic stimulation is in contrast to our results. Upon application of a norepinephrine reuptake inhibitor, C-LTMR recruitment significantly decreased, and this effect was maintained during application of three increasing doses of norepinephrine. These inconsistent findings could be due to a number of factors. First, the studies showing increased activity were conducted in cat and rabbit, and were recording spontaneous firing of afferents, whereas our study utilized optogenetic recruitment and assessed changes in recruitability. It is possible that the endogenously available NE levels that we increased via reuptake inhibition did not originate from sympathetic terminals, but from other

sources. The design of our study does not allow us to determine the origin of NE in the periphery; however, it does confirm that NE is endogenously available at hair follicles, and that it can influence the activity of C-LTMRs. Subsequent studies could incorporate sympathetic stimulation with selective C-LTMR recruitment to determine if the actions we see are related to sympathetic adrenergic input. Since we did not find an interaction between NE and SCI, it remains unclear if SCI alters levels or function of adrenergic signaling in the periphery. This is an area that requires further exploration, particularly because it is clear that SCI leads to perturbations in sympathetic function, and sympathetic drive is linked to changes in C-LTMR activity.

Serotonin

Evidence for serotonergic innervation at hair follicles is limited, although research has suggested that it may be released by Merkel cells[64]. Further sources of cutaneous serotonin include mast cells[77] and melanocytes[78]. To our knowledge the effect of serotonergic modulation on C-LTMRs has never been studied. Upon application of the serotonin reuptake inhibitor, we saw reduced recruitment in C-LTMRs. This confirms that serotonin is endogenously present at hair follicles, and furthermore that it can exert actions on these afferents. Reduced recruitment was maintained throughout direct serotonin application. There appeared to be dose-dependent reduction in the response magnitude of C-LTMRs such that higher concentrations of serotonin resulted in increased suppression; however, this effect was not significant. Interestingly, there was not a recovery in recruitment levels following washout. This could indicate that serotonin has long-lasting effects. However, given that there was a time-dependent reduction in overall C-LTMR recruitment, we cannot exclude the possibility that the lack of recovery after washout is simply due to unknown factors that reduce C-LTMR recruitment over the duration of the experiment. One interesting mechanism by which serotonin might influence C-LTMRs is via inflammation. The role of serotonin in inflammatory processes

is well documented[80, 81], and it can act as a pro-inflammatory agent, contributing to inflammation-driven hypersensitivity and hyperalgesia after nerve injury[82, 83]. We did not detect an interaction between serotonin and spinal cord injury; however, the relationship between SCI, inflammation, serotonin, C-LTMRs, and neuropathic pain is an intriguing subject requiring further study. If SCI-induced inflammation results in increased levels of serotonin in the periphery, and serotonin suppresses the activity of C-LTMRs, this could further support the theory that allodynia is due to a loss of the normally pain inhibiting role of C-LTMRs[20]. It should be noted that inflammation following SCI typically abates after the first couple of weeks[100] after injury, and our studies were conducted at a later time point, so any changes we observed would not be directly due to SCI related inflammation.

Acetylcholine

Sources of acetylcholine in the periphery include keratinocytes, lymphocytes, and melanocytes[42, 63]. Research has identified cholinergic actions on C-nociceptors, and one study found that an acetylcholine analogue carbachol did not induce changes in Aβ- or Aδ-LTMR activity[70], but to our knowledge ours is the first study to examine the effects of cholinergic modulation on C-LTMRs. We did not find any significant changes in C-LTMR recruitment in response to the nicotinic agonist, the cholinesterase inhibitor, nor acetylcholine itself when all animals were grouped. However, these results must be interpreted with caution due to the previously discussed discrepancies in the cholinergic paradigm. When we looked specifically at the group of animals that did not receive the nicotinic agonist, but rather only received the cholinesterase inhibitor, we did see significant reduction in response amplitude. These results indicate that endogenously available acetylcholine can reduce the activity of low threshold mechanoreceptors in hairy skin. It is possible that there was some interaction between the nicotinic agonist and the cholinesterase inhibitor, it is more likely that the group that received the nicotinic agonist simply had higher variability and we were therefore unable to

detect an effect. Future studies should be conducted which replicate our experiments with a higher number of animals, and consistent drug protocols. Additionally, it would be interesting to utilize selective muscarinic versus nicotinic antagonists in conjunction with acetylcholine to understand the specific mechanism of action, if any, on C-LTMRs.

Neuromodulation of LTMRs

C-low threshold mechanoreceptors are of particular interest due to their purported role in tactile allodynia. But other low threshold mechanoreceptors could well be involved in altered peripheral signaling. As discussed previously, peripheral signaling may play a role in maladaptive processes after injury[38-40]. One potential means of altered peripheral signaling is neuromodulation. In the following sections we will discuss three neuromodulators, norepinephrine, serotonin, and acetylcholine, and their effects on LTMRs. To determine the actions of these neuromodulators on LTMRs, we used air puff stimulation at varying forces to recruit C- Aδ- and Aβ-LTMRs. Due to the nature of our experimental approach and data analysis, we are unable to precisely discriminate between classes of LTMRs. Our analyzed data represents the summed barrage of recruited afferents to a given force, which is a physiologically relevant representation of the information that would project to spinal systems for subsequent integration and processing. Therefore it facilitates an important understanding of the overall impact of neuromodulation of sensory processing. Nonetheless, future analysis of this data via spike sorting to identify individual classes of afferents would provide further understanding of precise mechanisms.

Norepinephrine

Sources of norepinephrine and their known actions in the periphery have been thoroughly discussed previously. Briefly, with regard to LTMRs, a likely route of adrenergic modulation of hairy skin LTMRs is via sympathetic fibers which are spatially associated with LTMR endings at hair follicles[45]. When the norepinephrine reuptake inhibitor was applied to

the bath we saw reduced activity at the lowest force, and again at a mid-range force. All other forces had a similar trend in depression, but did not reach significance, which is most likely due to the high amount of variability in response magnitude across animals. Consistent with the optogenetic experiments, this confirms that norepinephrine is endogenously available and exerts its actions on LTMRs. Application of NE itself appeared to further suppress the response magnitude of recruited afferents, although this effect was not significant. Further research is needed to determine if a dose-dependent action exists and if so, if it is attributable to suppression of a greater number of individual units, or selective suppression of a class of afferents. The latter could be an intriguing mechanism by which norepinephrine can modulate the peripheral actions of one afferent class over another. Perturbations in the normal levels of NE, perhaps as a result of dysfunctional sympathetic output after SCI, could contribute to altered peripheral signaling.

Serotonin

Sources and actions of serotonin in the periphery have been discussed previously, but the most likely source relevant to LTMRs is from Merkel cells associating with hair follicles. One study has specifically demonstrated that release of serotonin from Merkel cells elicits action potentials from Aβ SA-LTMRs[64]. Here, we demonstrate that increasing endogenously available levels of serotonin can suppress the overall response of LTMRs to mechanical stimulation. Direct administration of serotonin also resulted in reduced response magnitude but did not appear to enhance suppression beyond levels seen with the reuptake inhibitor. Suppression was most prominent at lower forces, which may indicate that serotonin preferentially modulates afferents with the lowest thresholds. It is interesting that the study mentioned above found that serotonergic release from Merkel cells induced afferent firing, whereas we observed an overall reduction in recruitment. It would be interesting to identify specific afferent responses to determine if serotonin might have differential actions on Aβ SA-

LTMRs versus other LTMRs. It is also possible that our serotonergic reuptake inhibitor increased bioavailability of serotonin released from mast cells[77] and melanocytes[78], altering the responses of LTMRs which might not be affected by Merkel cell actions. Mast cells are involved in inflammatory processes, and serotonergic involvement in inflammation is well-documented [80, 81]. Inflammation may therefore influence the firing properties of LTMRs via serotonergic modulation. We did not examine any interactions between SCI and LTMR recruitment, but given the propensity for inflammation following SCI this would be an important relationship to study further.

Acetylcholine

As mentioned previously, acetylcholine is available in the periphery from keratinocytes, lymphocytes, and melanocytes[42, 63]. To our knowledge this project is the first to study its actions of LTMR afferents. Overall afferent responses were not altered by either the nicotinic agonist or the cholinesterase inhibitor. However, our experimental protocol and low number of animals may have made it difficult to detect an effect, and indeed at some forces we did observe a moderate, albeit insignificant, reduction in response magnitude. Future studies are needed to determine if an effect exists. We did observe a significant reduction in response upon application of acetylcholine, but only at 4mN or the three highest forces. There are several explanations for why we saw a significant response with acetylcholine but not the others. First, there were a higher number of animals that all received acetylcholine. Second, our recruited afferents may not express nicotinic, but rather muscarinic receptors and therefore the nicotinic agonist did not alter responses, but acetylcholine, which can act on both receptor types, did. Third, there was not an endogenous source of acetylcholine in our skin-nerve prep, and so the cholinesterase inhibitor did not alter LTMR responses. The actions of acetylcholine on our afferent response magnitude indicate that LTMRs may express some type of cholinergic receptor and therefore it seems likely that there would be an endogenous source of acetylcholine in the

skin. Lymphocytes produce acetylcholine, and this could serve as an additional way in which inflammation mediates afferent signaling. Future studies are certainly needed to replicate our experiments with consistent protocols and an increased number of animals. Furthermore, the interaction between SCI, inflammation, and cholinergic modulation of LTMRs should be further explored.

Potential limitations

There are several potential limitations to this study which must be addressed. First, due to the nature of the surgical procedure and the setup of the recording chamber, it was necessary to shave the area of skin that comprised the skin-nerve prep. As discussed previously, Aβ RA-, Aδ-, and C-LTMRs are all responsive to hair deflection. The mechanism of signal transduction is not well understood, but movement of the hair shaft is thought to elicit afferent firing via a physical connection between hair follicle epithelial cells and the lanceolate endings of LTMRs[89]. Therefore, in the absence of a hair shaft, our air stimulation protocol may not have been as effective at recruiting these afferents as it would have been with intact hairs. Second, given the low number of animals used in our study, and a great degree of variability in response magnitude across animals, it is certainly a possibility that effects were present but simply undetectable, particularly with regard to differences in SCI animals versus the control groups. As seen in all figures, standard error bars are rather large, and as such lack of significance observed in several instances may be due to an underpowered data set. Nonetheless many observations are highly significant and commonly those that are not have similar trends supporting a greater breadth to the observations. This is particularly evident in the acetylcholine group. Due to oversight in the continuity of our experimental protocol, the acetylcholine paradigm was inconsistent between the naïve group and the SCI/sham groups. The latter groups received epibatidine, a selective nicotinic agonist, prior to application of the cholinesterase inhibitor or acetylcholine. This agonist could well have long-lasting effects that may have

influenced the responses of afferents during the subsequent drug administrations. The naïve group did not receive epibatidine. In the interest of reaching a sufficient N, we chose to pool the three groups despite this discrepancy. Therefore, all acetylcholine results should be interpreted with caution and future studies will need to be conducted with consistent protocols. Third, as discussed previously, we noted a time-dependent reduction in evoked responses over the course of the experiment, and washouts were not sufficient to return activity to baseline (as exemplified by Figure 14). This was not due to issues with suction quality or overall prep viability, as evidenced by the consistent magnitude of spontaneously active units over the course of the experiment. It could be due to fatigue in recruited units, or long-term effects of the drug that were not mitigated by washout. Our study did not attempt to distinguish between these two possibilities. This could be accomplished in the future by replicating the length of time and stimulation parameters of our experimental protocol, but without any drug additions. Finally, two different mouse lines were used in this study, as it was conducted in collaboration with the Garraway lab. In theory, these lines should both selectively activate TH+ C-LTMRs, but we cannot exclude the possibility that there are differences in the baseline response properties of these mice. Furthermore, since the naïve animals came from one line while the SCI groups came from another, it would be unwise to draw conclusions about differences between SCI and naïve animals.

Future Directions

This study was a preliminary examination of the effect of neuromodulation on LTMRs, and provides evidence that norepinephrine, serotonin, and acetylcholine can modulate the responses of low threshold mechanoreceptors. This opens up a huge number of possible avenues of future research into the role of neuromodulation in altered peripheral processing.

The nature of our analysis of the air stimulation protocol did not allow us to detect specific afferents. Since the data has already been collected a natural first step to further

understand the specific modulatory effects on each LTMR would be a more detailed analysis of the data. In our protocol air stimulation was delivered for a full second. Since each class of LTMR has distinct adaptation characteristics, future analysis could look at specific regions within the one second pulse of air stim. For example, Aβ RA-LTMRs, as the name indicates, are rapidly adapting afferents with known conduction velocities; therefore, their responses would arrive within a specific window, and would be short-lived. Future analysis could focus on that window and would provide a more specific understanding of how norepinephrine, serotonin, and acetylcholine affect their activity. The same analysis could be applied to the other four LTMRs that innervate hairy skin. A more detailed analysis using spike sorting could identify groups of units with similar waveforms, providing an additional level of specificity.

In this study, in both the mechanical and optogenetic protocols, we began our assessment of neuromodulation with a reuptake or cholinesterase inhibitor to assess endogenous availability of each neuromodulator, followed by the neuromodulator itself at varying doses. Subsequent studies could assess the actions of selective receptor agonists to better understand the specific mechanisms of action and how they might influence the activity of selective classes of afferents.

There are two known outcomes of SCI that may contribute to neuromodulation and altered peripheral signaling: sympathetic disruption and inflammation. Sympathetic fibers are predominantly adrenergic, and we have now provided evidence that the presence of norepinephrine in the skin can alter the activity of C-LTMRs and other LTMRs more broadly. Future research is needed to determine if sympathetic firing (a) releases norepinephrine at the hair follicles, and (b) leads to changes in the activity of any or all LTMRs. It would be particularly interesting to link SCI-related changes in sympathetic firing to changes in peripheral signaling, especially if those changes were in C-LTMRs and were accompanied by mechanical hypersensitivity. Studies such as this could provide the first direct link between SCI mediated allodynia and altered peripheral processing in C-LTMRs. Inflammation is another

route by which SCI might lead to altered activity in cutaneous afferents. We have discussed the involvement of both serotonin and acetylcholine in inflammation, and demonstrated that they can alter the responses of LTMRs. However, our study does not determine precisely where either neuromodulator might be originating from. Potential avenues of research in this area include inducing inflammation to assess LTMR responses, and measuring levels of acetylcholine and serotonin at the hair follicle. Additionally, there are a great many other potential modulators in the skin milieu. Many of these are substances released during inflammatory processes, and their actions on LTMRs remain unstudied.

Finally, an interesting direction of study might involve replicating these studies in an *in-vivo* model. In order to assess the relationship between altered peripheral signaling and any pain states, it is imperative to assess behavioral changes. We have shown that norepinephrine, serotonin, and acetylcholine can alter the response properties of LTMR, but our experimental protocol cannot relate those changes to any meaningful changes in perception or behavior. Therefore, an intriguing avenue of research could involve assessment of behavioral changes in response to neuromodulation in the periphery, particularly in spinal cord injured animals. Taken together, this research could further our understanding of the complex relationship between SCI, neuropathic pain, and the contributions of altered peripheral signaling.

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