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Behavioral Insights Into Nociceptor Function: A Systematic Approach To Understanding Postsurgical And Neuropathic Pain Mechanisms In Rats

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BEHAVIORAL INSIGHTS INTO NOCICEPTOR FUNCTION: A SYSTEMATIC APPROACH TO UNDERSTANDING POSTSURGICAL AND NEUROPATHIC PAIN MECHANISMS IN RATS

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A

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By

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Houston, TX
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I dedicate this body of work to my mother and father,

and to myself for not giving up.

“The literature of the world should be open to him – nothing prohibited, sealed or hidden. No subject can be too sacred to be understood.” - Robert G. Ingersoll, *The Truth*, 1896

Acknowledgements

I want to first acknowledge my mother and stepfather for supporting me these past 8 years while I have been in grad school. You were not always able to solve my problems, but you listened. Sometimes listening is all that is necessary, so thank you. I also want to acknowledge my late father for raising me to be the person I am today. To my parents I owe my perfectionist nature and unyielding integrity.

To the friends I have gained while in grad school, thank you. You listened, you argued back, you reined me in sometimes, and you helped me find solutions I was sometimes too blind to see. I have made it this far because of the support system you each helped provide.

To all of my coworkers, collaborators, and graduate committee, thank you for working with me (I know I can be difficult), challenging me, and providing critical feedback. It is my hope this dissertation reflects not only my efforts but each of your own.

I owe a great deal to my advisor, Dr. Terry Walters. Your critical mind and approach to research has helped me to develop into the scientist I am today; I have always found our discussions (and disagreements) to be intellectually fruitful. Your patience and willingness to let me fail and learn from my mistakes have been crucial to my training. Thank you for the opportunity to be a member of your lab and for mentoring me.

Abstract

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Max Allen Odem, M.S.

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Postsurgical and neuropathic pain are each clinically common, and often associated with ongoing pain. Ongoing pain has been linked to ongoing activity (OA) in human C-fiber nociceptors. Preclinical studies using rodent neuropathic models have concentrated on allodynia driven by OA generated in non-nociceptive Aβ fibers, but little attention has been paid to postsurgical pain in sham controls or to C-fiber nociceptor OA promoting ongoing pain.

Operant assays that reveal negative motivational and cognitive aspects of voluntary pain-related behavior may be particularly sensitive to pain-related alterations. In the mechanical conflict (MC) test, rodents can freely choose to escape from a brightly lit chamber by crossing sharp probes. Most studies employing the MC test habituate rodents to the device and measure the latency to escape the bright light. We found reducing habituation caused rats to repeatedly return to the light chamber when probes were absent, presumably as part of their exploratory behavior. We asked whether combining motivations to avoid the bright light and to explore the device would reveal a conflicting, pain-related reluctance of rats to cross noxious probes. Rats with a thoracic spinal cord injury (SCI), lumbar spinal nerve transection, or chronic constriction injury of the sciatic nerve, as well as their sham controls, exhibited heightened pain-avoidance behavior compared to uninjured controls. These findings have important implications for investigations into behavioral and neuronal alterations contributing to postsurgical and neuropathic pain.

Many C-fiber nociceptors generate OA *in vivo* in rats with SCI and ongoing pain. Probable nociceptors continue to generate OA *in vitro* after dissociation. We used whole-cell recordings from isolated dorsal root ganglion neurons and novel algorithms that analyze irregular changes in membrane potential (MP) to define neurophysiological alterations underlying SCI-induced nociceptor OA. In a distinct type of probable nociceptor, SCI caused 3 chronic alterations that promote OA: 1) depolarization of resting MP, 2) reduction in the voltage threshold for action potential generation, and 3) enhancement of depolarizing spontaneous fluctuations (DSFs) in MP. *In vitro* modeling of acute inflammation by combining serotonin with artificial depolarization also potentiated DSFs and OA. These findings reveal nociceptor specializations for generating OA during ongoing pain.

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Frequently Used Abbreviations

5-hydroxytryptamine, serotonin (5-HT)

Action potential (AP)

Basso, Beatie, Bresnahan locomotor rating scale (BBB)

Chronic compression of DRG (CCD)

Chronic constriction injury (CCI)

Conditioned place preference (CPP)

Dorsal root ganglia (DRG)

Depolarizing spontaneous fluctuation (DSF)

Hyperpolarizing spontaneous fluctuation (HSF)

Isolectin B4 (IB4)

Mechanical conflict (MC) and system (MCS)

Mas-related G protein-coupled receptor member D (Mrgprd)

Nonaccommodating (NA)

Ongoing activity (OA)

Peripheral nerve injury (PNI)

Partial sciatic injury (PSI)

Paw withdrawal threshold (PWT)

Rapidly accommodating (RA)

Resting membrane potential (RMP)

Spontaneous activity (SA)

Spinal cord injury (SCI)

Spared nerve injury (SNI)

Spinal nerve ligation/axotomy (SNL/SNA) and modified SNA (mSNA)

Spinal nerve transection (SNT)

Transient receptor potential vanilloid-1 (TRPV1)

Preface

I joined the lab of Edgar T. Walters, Ph.D. because he and I have broad, mutual interests in animal behavior, nociception, and pain from an evolutionary perspective. My personal goal from the beginning has been to better characterize the negative affective and motivational components of pain in rats with spinal cord injury (SCI) to better understand their experiences and to develop a deeper appreciation of their sacrifice for human benefit. I began my research using operant behavioral tests that assess evoked and ongoing pain, and I gained an interest in using *in vitro* whole-cell patch electrophysiology to study the neurophysiological basis of nociceptor activity that may translate at the behavioral level to ongoing pain. Ultimately, everything we study in preclinical pain research *must* make sense at the behavioral level at some point. Some of my behavioral and electrophysiological experiments cover topics that currently lack well-defined outcome measures. Therefore, I have adopted a descriptive approach in combination with using some novel methodologies and analytical tools in hope of establishing a solid foundation upon which future mechanistic predictions can be made. Descriptive science matters [144], and any predictions about pain-related mechanisms are only as powerful as our descriptions of pain behavior and nociceptor function.

For continuity, I have decided to first present my behavioral study using a recently developed operant mechanical conflict (MC) test to reveal postsurgical effects of sham procedures on evoked pain and avoidance behaviors. My behavioral experiments using the conditioned place preference (CPP) test to reveal SCI-induced ongoing pain will not be the primary focus, but some of the work will be referenced (see [389]) and shown when appropriate. This will be followed by my electrophysiology study using whole-cell recordings of nociceptors *in vitro* and novel algorithms to characterize the neurophysiological basis of ongoing activity associated with ongoing pain. This reflects my mentality that one ought to first be able to describe pain-related behavior before trying to attribute it to potential underlying mechanisms.

Chapter 1: *The fundamental unit of pain is assessed behaviorally*

Contrary to the proclamation of Reichling *et al*. in their 2013 review in the journal *Pain* [300], the fundamental unit of pain is *not* the cell. I think this reductionist perspective has been integral to the overwhelming expansion in knowledge of mechanisms associated with pain, but it simultaneously diminishes the importance of the means by which those with pain show it and how researchers ultimately must assess it (i.e., behaviorally). Pain-related behavior is not the mere result of a few mechanisms of interest. Indeed, there is a wide disconnect (e.g., "valley of death", see [118,370,390,392]) between preclinical mechanistic research using laboratory animals and the successful translation of clinical therapeutics for pain. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential damage, or described in terms of such damage". This most widely accepted definition of pain is predicated upon the notion that pain is emergent. I think the nested model for the universe of pain proposed by J.D. Loeser [228] makes this point in the simplest manner possible (**Fig. 1**). In his model the second principal component of pain is perception, which is largely dependent upon a great multitude of molecular and cellular processes that underlie the first principal component, nociception. This is important to distinguish, nociception is not pain and the two are not interchangeable. Nociception is the body's detection system for damaging and/or potentially damaging stimuli. Pain can be perceived in the absence of nociception, and a noxious stimulus can activate nociceptive systems without being perceived as painful [129]. Typically, once a noxious stimulus is transduced in the peripheral nervous system (PNS) by nociceptors (neurons specialized for responding to noxious stimuli) and integrated in the dorsal horn of the spinal cord, the encoded sensory information is relayed to higher-order processing centers (i.e., pain matrix) in the central nervous system (CNS) for perception. The third principal component in Loeser's model that follows pain perception is suffering. Loeser broadly defines suffering as the affective response to any psychosocial constructs (e.g., fear, depression) which can influence

the valence attributed to pain. For a review of psychosocial constructs that influence pain and expansions upon Loeser's principal components see [112,245]. Any perceived threat to a person's or animal's integrity is likely to contribute to suffering [59]. Loeser emphasizes that these three principal components (nociception, pain perception, and suffering) are experienced internally and therefore cannot be explicitly quantified nor validated. This is especially true in laboratory animals, they cannot directly communicate with researchers and we ought to meticulously observe their behavior in an attempt to draw plausible inferences about their experiences. Loeser makes this point very clear when he states that the objectively real, quantifiable sum of nociception, pain perception, and suffering is pain behavior, the final all-encompassing piece in his model. In light of Loeser's perspective, it is my interpretation that the fundamental unit of pain is not the cell, it is the amalgamation that is a person, an animal. Any mechanisms that potentially underlie and describe the nature of nociception, the perception of pain, and the emotional consequences thereof *must* be able to reasonably explain for and/or predict observable changes in behavior. Thus, the tests that pain researchers have designed to study pain-related behavior in animals are the crucibles by which mechanisms can be discovered, refined, and… set aside for later consideration. To be clear, I do not think the behavioral tests that are currently available are the single points of failure that solely explain why there is this "valley of death", but their application and whether or not the behaviors being measured are as informative as originally considered have been questioned [255,351].

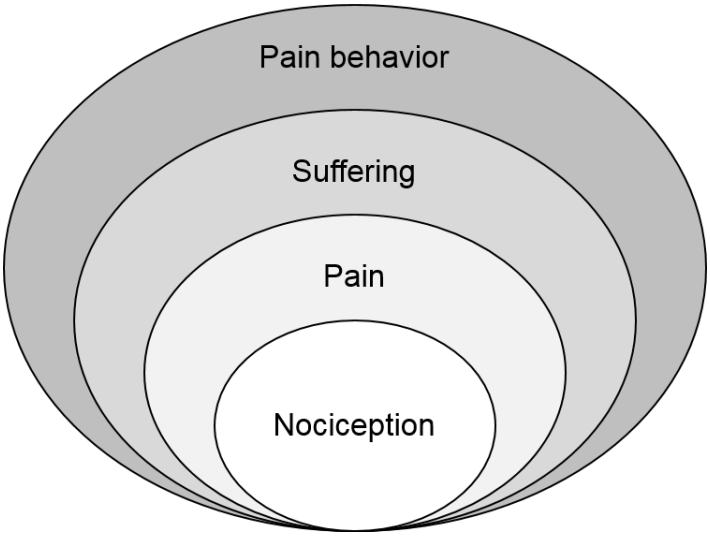


Figure 1. A nested model for the universe of pain, proposed by J.D. Loeser, M.D. Colors adapted from Figure 1 in [228] and reprinted with permission under license. Title: Pain and Suffering; Author: John Loeser; Publication: Clinical Journal of Pain, The; Publisher: Wolters Kluwer Health, Inc.; Date: June 1, 2001. Copyright © 2000, © 2000 Lippincott Williams. License number 4443780134967 granted to Max A. Odem on October 7, 2018.

1.1. In the beginning there were only reflex tests…

To be considered an appropriate, potentially translatable animal model for pain, a model should recapitulate the pathophysiology of a clinical condition of interest and it should present symptoms and signs that mirror clinical manifestations of pain. Behavioral outcome measures should be feasibly obtainable, objectively interpretable, replicable across research groups, and most importantly be assessed under strict ethical and humane guidelines. Researchers have a plethora of behavioral tests [21,140,141,344,352] at their disposal for studying the many facets of pain. Furthermore, there is a diverse collection of animal models that recapitulate a broad spectrum of clinically relevant pain-related conditions, injuries, and disease states [21,32,40,68,140,141,164,182,197,199,238,361]. Elucidating how mechanisms underlying pain behavior converge and diverge in many different animal models has been crucial for identifying some new and potentially successful treatment strategies [33,79,187]. Despite many options

and advancements, the preclinical side of pain research has been stymied due to multiple factors [253,351,390,392]. Mogil and Crager identified one factor early on; in only 4 years (2000 to 2004) 90% of the 259 studies published in *Pain* that utilized behavioral assessments of inflammatory or neuropathic pain relied solely upon reflex tests that measure rodents' sensitivity to evoked stimuli, typically mechanical or thermal in nature [255]. Mogil and Crager also astutely point out the disparity between the dogmatic-like use of reflex tests for nociception and growing numbers of preclinical rodent models of neuropathic, inflammatory, postsurgical, and other painful conditions. Vierck *et al*. also state very clearly "pain is not a reflex" as they echo a growing call to expand our understanding of the motivational and emotional consequences of pain [351]. I think the value of behavioral tests currently available to pain researchers ought to be reassessed according to their descriptive power, and a greater emphasis should be placed on tests that might characterize animal models and conditions based upon multidimensional components of pain [256,286].

1.2. Overview of tests of pain-related behaviors in rodents

Loeser broadly defined the complexities of pain behavior [228] while others [112,245] have expanded upon his definitions to identify more intricate interactions among a breadth of psychosocial constructs. Here I will suggest how Loeser's principal components and three other characteristics can be applied to subdivide many of the behavioral tests used today. I think doing so provides insight into the theoretical validity, descriptive power, and dependability of each test. As will be evident, the use of multiple overlapping and non-overlapping tests based on different principal components ought to improve the predictive validity of experiments and help studies arrive to stronger conclusions about any pain-like states and treatment-related effects; studies that do so are potentially more informative [140].

I posit the following non-exhaustive list (see **Table 1** and references in the legend) of common behavioral tests for rodents and key test characteristics by which I think they should

be delineated (for broader review see [21,140,344,387]). The first characteristic is Loeser's principal component for pain behavior. To reiterate, the three principal components are nociception, pain perception, and suffering [228]. To be effective at revealing some aspect of pain behavior, a behavioral test should yield results that pertain to at least one or more of the following: 1) evidence of nociceptor activation, 2) evidence of motivational states and/or non-reflexive pain-directed behaviors that necessitate higher-order processing and would suggest pain perception, and 3) evidence of altered emotional states (e.g., anxiety- and depression-like behaviors) that might imply suffering (note: a single test does not quantify suffering, but a collective profile of altered emotional states might reasonably imply the presence of suffering). The type of behavior that is elicited is also critical; for the second characteristic I note whether the rodent is permitted to behave independently under its own volition or is purposefully restrained for response evocation (i.e., voluntary or involuntary behavior). The third characteristic is whether or not an evoked stimulus is necessary to assess the behavior of interest, and if so the nature of the stimulus. To clarify, no external stimuli are applied to rodents in the gait analysis/weight bearing test, but the rodents' own movement and substrate may be sufficiently painful (see [30]). Finally, in an effort to identify potential sources of unconscious bias, I have listed if a test requires any direct human interaction. This does not refer to general handling procedures before/after a test is completed, it is specifically in reference to circumstances in which an investigator must evoke a behavioral response to record the outcome (e.g., hand-held delivery of von Frey filaments). In sum, **Table 1** reviews pain-related behavioral tests with regards to 4 major categories: 1) tests of involuntary reflexes in response to external stimuli, 2) tests of voluntary behaviors in response to external stimuli, 3) tests of ongoing/spontaneous pain in the absence of external stimulation, and 4) tests designed to identify affective disorders and phenotypes.

Name of test/Reference	Test characteristics			
	Loeser principal component	Type of behavior	Stimulus-evoked/Modality	Human interaction necessary
Hot/cold plate[23]	Nociception	Involuntary	Yes – Thermal	No
Acetone[4]	Nociception	Involuntary	Yes – Thermal	Yes
Tail flick[7]	Nociception	Involuntary	Yes – Thermal	Yes
Hargreaves[11]	Nociception	Involuntary	Yes – Thermal	Yes
von Frey[3]	Nociception	Involuntary	Yes – Mechanical	Yes
Randall-Selitto[21]	Nociception	Involuntary	Yes – Mechanical	Yes
Needle/Pin-prick[22]	Nociception/Pain perception	Involuntary/Voluntary	Yes – Mechanical	Yes
Formalin[9]	Nociception/Pain perception	Involuntary/Voluntary	Yes – Chemical	No
Operant thermal escape[18]	Nociception/Pain perception	Voluntary	Yes – Thermal	No
Operant mechanical conflict[17]	Nociception/Pain perception	Voluntary	Yes – Mechanical	No
PEAP[15]	Nociception/Pain perception	Voluntary	Yes – Mechanical	Yes
CPA[12]	Nociception/Pain perception	Voluntary	Yes – Chemical	No
Gait analysis/weight bearing[5]	Nociception/Pain perception	Voluntary	Unclear	No
Ultrasound vocalization[2]	Pain perception	Involuntary/Voluntary	No	No
Facial grimace[16]	Pain perception	Involuntary/Voluntary	No	No
CPP[14]	Pain perception	Voluntary	No	No
Burrowing[8]	Anxiety-like behavior	Voluntary	Yes – Mechanical	No
Marble burying[1]	Anxiety-like behavior	Voluntary	Yes – Mechanical	No
Light/dark[6]	Anxiety-like behavior	Voluntary	No	No
Open field[14]	Anxiety-like behavior	Voluntary	No	No
EPM/EZM[19]	Anxiety-like behavior	Voluntary	No	No
Sucrose preference[13]	Depression-like behavior	Voluntary	No	No
Forced swimming[20]	Depression-like behavior	Voluntary/Involuntary	No	No

Table 1. Non-exhaustive list of pain-related behavioral tests for rodents. References (superscript corresponds to the table, number in brackets corresponds to bibliography): [1]Broekkamp *et al*., *Eur J Pharmacol*, 1986 [45]; [2]Calvino *et al*., *Neuroreport*, 1996 [51]; [3]Chaplan *et al*., *J Neurosci Methods*, 1994 [63]; [4]Choi *et al*., *Pain*, 1994 [393]; [5]Clarke *et al*., *Physiol Behav*, 1997 [76]; [6]Crawley and Goodwin, *Pharmacol Biochem Behav*, 1980 [80]; [7]D'Amour and Smith, *J Pharmacol Exp Ther*, 1941 [84]; [8]Deacon, *Nat Protoc*, 2006 [87]; [9]Dubuisson and Dennis, *Pain*, 1977 [110]; [10]Hall, *J Comp Physiol*, 1934 [150]; [11]Hargreaves *et al*., *Pain*, 1988 [152]; [12]Johansen *et al*., *Proc Natl Acad Sci U S A*, 2001 [178]; [13]Katz, Pharmacol Biochem Behav, 1982 [185]; [14]King *et al*., *Nat Neurosci*, 2009 [192]; [15]LaBuda and Fuchs, *Exp Neurol*, 2000 [204]; [16]Langford *et al*., *Nat Methods*, 2010 [208]; [17]Lau *et al*., *Neurorehabil Neural Repair*, 2012 [210]; [18]Mauderli *et al*., *J Neurosci Methods*, 2000 [241]; [19]Pellow *et al*., *J Neurosci Methods*, 1985 [280]; [20]Porsolt *et al*., *Nature*, 1977 [287]; [21]Randall and Selitto, *Arch Int Pharmacodyn Ther*, 1957 [295]; [22]Seltzer *et al*., *Pain*, 1990 [319]; [23]Woolfe and MacDonald, *J Pharmacol Exp Ther*, 1944 [373]. CPA, conditioned place avoidance; CPP, conditioned place preference; EPM, elevated plus-maze; EZM, elevated zero-maze; PEAP, place escape/avoidance paradigm.

1.2.1. Notable limitations associated with involuntary reflex tests

A majority of the standard reflex tests are limited to measuring involuntary withdrawals of the paws or tail to an applied mechanical or thermal stimulus. They can potentially reveal alterations in sensory and nociceptor properties that manifest both peripherally and in the dorsal horn (for review [21]), but flexion does not necessitate cognition and stimuli may not be consciously perceived (see [240]). Additional limitations of reflex tests pertain to methodology, testing consistency, and reliability of the results. Rodents often require acclimation to testing facilities and researchers [334] as well as laborious baseline testing procedures, re-testing, and detection of potential outliers. Another important requirement of these tests is that stimuli are evoked in a consistent, repeatable manner and rodents are treated equally. In other words, rodents are often restrained either by hand or in small, usually clear acrylic, chambers and there is some form of human interaction with the rodent. This is an opportunity for unknown sources of bias to negatively impact data recording [38], so the exclusive use of reflex tests ought to be met with caution.

As an example, the von Frey test is one of the simplest, most efficient tests for assessing mechanical nociception. Each von Frey filament is calibrated to bend at a specific gram force and are hand applied to the plantar surface of the paws; filaments range from <1 gram to >100 grams. Unfortunately, there is no agreed upon standard method using von Frey filaments (e.g., see Table 1 in [90]). Not all groups use the same range of filaments, starting filament, number of stimuli, stimulus duration (e.g., some groups press filaments for ~1 second while others press 4-5 seconds; see [58,90]), or threshold calculation methods (e.g., original Chaplan/Dixon “up-down” method [63,98,99] versus reduced procedure [37]). Finally, many studies report absolute thresholds in grams using aforementioned calculation methods, but Mills *et al*. point out many studies do not use log transforms of the data to account for Weber’s law [251]. Proper representation is necessary to identify meaningful treatment-related effects (e.g., see [251]). Incomparable results between studies may be due methodological differences

[38] (see also [97] for Hargreaves radiant heat test) combined with differences in mechanisms associated with pain wind-up (for review [162]). The von Frey test is also a poor approximation of human pain test conditions [352]. It is possible the von Frey test preferentially recruits myelinated, fast conducting A-fibers without recruiting unmyelinated, slowly conducting C-fibers [36], suggesting it may not be effective at modeling some forms of pain.

1.2.2. Tests of voluntary behavior offer greater insight into multidimensional components of pain

Under typical conditions the needle/pin-prick and formalin tests require that rodents be restrained, but the noxious stimuli used promote “spontaneous” pain behaviors that may be voluntary and reflect cognitive processing. Reflex intensity and hyperalgesic behaviors are distinguished using qualitative descriptions (e.g., prolonged withdrawal, excessive grooming of stimulated paws). In the needle/pin-prick test a rodent’s paws are quickly probed using a sharp needle/pin. Under naïve conditions the test elicits typical rapid withdrawal reflexes, but under some potentially painful conditions rodents elicit exaggerated withdrawals and behaviors such as limb guarding, licking, and vocalizations [165]. In one test variant rodents learn to passively avoid noxious stimulation of an injured paw (see [375]), suggesting awareness. Similar hyperalgesic behaviors are observed when formalin is injected into a rodent’s paw; behavioral assessments are made during early and late phases of “spontaneous” pain. Other inflammatory substances can be used in place of formalin (e.g., complete Freund’s adjuvant, CFA; serotonin, 5-hydroxytryptamine, 5-HT).

Considerations for rodent autonomy during development of new behavioral read-outs may help improve the predictive value of preclinical models of pain for translational drug discovery [32]. Indeed, newer, more sophisticated behavioral tests have been specifically designed to take advantage of the innate preferences (e.g., exploration of novel environments [80,113]) and stock behaviors (i.e., naturally occurring in the wild) of freely behaving rodents

(for review [140,344,387]), and to remove human sources of bias. These voluntary tests are gaining tremendous traction as effective, automated tools for identifying the negative affective-motivational components of pain that are particularly important clinically [256,286,344,351,352,392]. Operant behavioral tasks such as the CPP test [19,85,192,264,265,291,389] and the place escape/avoidance paradigm (PEAP) [15,41,124,204–206,375] can capture persistent, aversive pain-like states and demonstrate how spontaneous pain influences decision-making [256,286]. Noxious sensory information may be self-evoked in some rodents with painful conditions (see [30]), so gait analysis/weight bearing tests might also reveal how ongoing pain can influence natural ambulatory movement.

Animal suffering may not be quantifiable and difficult to qualitatively describe without using anthropomorphic terms, but the reality is laboratory rodents exhibit primal emotional states, complex signs of empathy, and pain-related distress (for review [248]). Meyza *et al*. [248] state that acknowledging the presence of empathy permits generation of animal models relevant to human conditions. Behavioral tests of altered emotional states like anxiety (e.g., open field, elevated mazes) and depression (e.g., forced swim, sucrose preference) have begun to reveal the emotional consequences of neuropathic pain in rodents [387]. High-frequency vocalizations and facial grimacing may confer to conspecifics distress due to ongoing/spontaneous pain, but this has not been adequately tested or ruled out (for review [254]). In their current forms, the marble burying and burrowing tests are used to assess anxiety, but I also interpret them to involve some degree of self-evoked mechanical stimulation. Burrowing is decreased in some painful conditions like nerve injury and SCI [11,41,232]. These studies do not directly determine whether burrowing is sensitive to above and at-level injury-induced hypersensitivity in the forelimbs. Rats with SCI exhibit robust above and at-level mechanical hypersensitivity [28,57,171], so it is plausible their performance in the burrowing test may not reflect an anxiety-like phenotype as much as sensitization to mechanical stimulation. Likewise, there is insufficient evidence with the marble burying test to distinguish

affective and nociceptive components. It is self-evident, but the forced swim test is pseudo-voluntary; rodents must swim to stay afloat inside an inescapable water-filled chamber. This test is predicted to model learned helplessness (i.e., coping) and depression-like behavior; under painful conditions rodents spend less time vigorously swimming while maintaining their head above water with minimal movement [387]. Despite the availability of many behavioral tests, the affective consequences of pathological forms of pain are still unclear [387].

1.3. Modeling clinically relevant neuropathic pain conditions in rats

In this section I want to narrow focus to neuropathic pain and briefly describe relevant patient conditions. I will then provide a brief overview of several rat models of surgically-induced neural injury used to study neuropathic pain. Finally, I will review some of the behavioral evidence for pain in these neuropathic models that has been extracted using many of the aforementioned behavioral tests.

Pain which originates from acute activation of nociceptors is referred to as nociceptive pain, but pain can also be inflammatory and/or pathological in origin. Neuropathic pain covers a range of disorders whose etiology stems from some primary damage and/or disease to regions of the PNS and/or CNS (for review [79,188]). Classifying neuropathic pain becomes extremely important when attempting to better understand the symptoms, underlying mechanisms, and pain associated with different etiologies. An important distinction in terminology must be established when describing pain as acute or ongoing/spontaneous. Ongoing pain can originate after extrinsic stimulation of nociceptors and/or activity generated in central circuits, or it can be truly spontaneous in origin. The terminology for the neuronal/nociceptor activity that underlies ongoing and spontaneous pain must also be distinguished. Ongoing activity (OA) is any continuous discharge of actions potentials (AP) due to extrinsic and/or intrinsically driven activation of the neuron/nociceptor. Spontaneous activity (SA) is a subclass of OA in which activity is solely generated due to intrinsic properties of the neuron/nociceptor, that can only be

reasonably demonstrated when the neuron/nociceptor is isolated. Things become increasingly more complex when one considers the type of pain sensation (i.e., modality); persons with neuropathic pain report many different sensations such as sharp pins and needles, radiant burning, a dull aching, etc. The predominant view is that many nociceptors are polymodal [23], meaning they can respond to multiple types of stimuli and encode for multiple sensations. Two common and troublesome symptoms associated with neuropathic pain are allodynia (i.e., an innocuous stimulus becomes painful) and hyperalgesia (i.e., a normally noxious stimulus elicits an exaggerated pain response). Clinical outcome measures for the intensity and degree of pain typically involve sensory assessments of allodynia and hyperalgesia [175]; which stimuli evoke which types of nociceptive responses. For the truly unfortunate, ongoing/spontaneous pain might not ever dissipate as it is potentially driven by peripherally and/or centrally generated OA and/or SA.

A recent meta-analysis of epidemiological studies estimates roughly 7-10% of the general population has neuropathic pain [160], but the prevalence rates vary per condition. For example, roughly 15% of those with diabetic neuropathy are predicted to have neuropathic pain, while amputees and persons with SCI may have some of the highest prevalence rates exceeding ~50% [188]. Furthermore, different neuropathic etiologies will exhibit vastly different sensory and pain profiles. Baron *et al*. used cluster analysis with a hypothesis-free approach (i.e., no assumptions about underlying mechanisms) to identify distinct sensory profiles in patient consortia with peripheral forms of neuropathic pain: polyneuropathy, peripheral nerve injury (PNI), postherpetic neuralgia, and radiculopathy [20]. Their cluster analysis reveals three major sensory profiles that span all four peripheral forms of neuropathic pain included in the study: sensory loss (42-53% of patients), thermal hyperalgesia (33%), and mechanical hyperalgesia (14-24%). The Baron *et al*. study reinforces the fact that not all neuropathic pain conditions are the same in all patients. There is a substantial amount of heterogeneity within and across conditions, each involving potentially distinct, complex sets of underlying

mechanisms [79]. Persons with persistent forms of neuropathic pain can suffer tremendously due to development of what is generally referred to as chronic pain (difficult to define, see [184] for review). Treatment options are sorely inefficient [120,390]. Besides pain, persons must also contend with associated comorbidities (e.g., affective disorders like anxiety, depression) which can develop and plague livelihoods [54,119,387]. For example, persons with SCI can exhibit varying degrees of above-level, at-level, and below-level pain, with multiple distribution patterns (e.g., diffuse, localized), spanning multiple sensory experiences (e.g., aching, burning, throbbing) for the rest of their lives [46,119,367]. Compared to the general population, persons with SCI are 3 times more likely to commit suicide [54]. These diverse conditions can be difficult to effectively model, no single rodent model exhibits all conditions that might be relevant clinically.

1.3.1. An overview of common rat models of surgically-induced neural injury

A myriad of rat neural injury models have been developed to study neuropathic pain-related behaviors and mechanisms. Some models are elegantly simple, requiring minimal surgical expertise, while others can be technically sophisticated and demanding. Each of the following models requires major surgery (i.e., extensive tissue dissection) and careful experimental considerations for postsurgical forms of acute and chronic pain ought to be taken (for review of postsurgical pain [64]).

A common form of peripheral neuropathic pain is radiculopathy (i.e., pinched nerve). This condition has been modeled using a steel rod to chronically compress the dorsal root ganglia (DRG) in the CCD model [167,233,333,400,405] and by rhizotomy of proximal dorsal/ventral roots in the spinal column [22,116,220,327,340]. Notably, the CCD and rhizotomy models require substantial damage to muscle and bone surrounding the spinal cord. Multiple SCI models have been designed to recapitulate more severe central injuries. The level of injury (e.g., cervical, thoracic) and severity of injury (e.g., complete transection, hemisection,

contusion) can be modified to best fit the patient population characteristics (e.g., level of pain) that are of interest to researchers. Some models are designed to reflect conditions in which the cord has been sectioned [71] or compressed [73,74], but contusive SCI models are considered to be the most clinically relevant [391]. Contusive SCI is performed by weight drop or controlled piston impact; most mild to moderate contusions are performed in the mid to lower thoracic regions [24,25,28,57,90,171,196,377,389]. Due to difficulties associated with using paralyzed rats (e.g., long recovery for behavioral testing, loss of neurogenic bladder function), a unilateral cervical contusive model has also been developed that may be better suited for early post-SCI behavioral testing and exercise rehabilitation studies [91,309].

Peripheral nerve injury models that reflect common forms of peripheral neuropathy are more widely used. The PNI models typically involve axotomy/ligation or constriction of the nerves and/or nerve branches innervating the hindlimbs; this typically provides critical within animal controls (e.g., uninjured limb contralateral to side of injury). There are several prominent PNI models, like the spinal nerve ligation/axotomy (SNL/SNA) injury in which the lumbar L5 and/or L6 nerves are ligated and axotomized [108,163,214,225]. Some groups transect L5 without ligation (spinal nerve transection, SNT) [338,339,345] while others tie additional loose ligatures around an uncut L4 nerve (modified SNA, mSNA) [105,215]. Moving distally, ligatures can be loosely tied around the sciatic nerve (chronic constriction injury, CCI) [13,31,77,108,181,193,214,242,350] or the nerve can be partially ligated/transected (partial sciatic injury, PSI) [108,214,319]. In the spared nerve injury (SNI) model 2 of the 3 sciatic nerve branches are axotomized [88,108,213]. Finally, miscellaneous complete nerve transections have also been used [108,249,354,395]. Among many benefits, the surgical procedures for these models are simpler and damage fewer peripheral tissues.

1.3.2. Behavioral evidence for pain-like states in rat models of neuropathic pain

After several decades of research, what is the collective behavioral evidence for pain-like states in rat models of surgically-induced neural injury? Two common symptoms (e.g., allodynia and hyperalgesia) reported by persons with neuropathic pain pertain to some underlying sensory dysfunction that causes modality-specific forms of hypersensitivity. Following review of the original descriptions of each rat model and multiple follow-up studies, it is evident a vast majority of evidence for pain-like states comes from reflex tests for nociception (see **Table 2** and references in the legend). All eight of the neural injuries described cause some form of mechanical and thermal (heat and/or cold) hypersensitivity, although some models have received less attention due to extenuating circumstances (e.g., limb autotomy; see rhizotomy and miscellaneous nerve transections). Despite the efficiency and widespread use of reflex tests, they do not accurately reflect all of the complex, multidimensional components of pain [255,351,352,392]. There exist other models/tests [21,140,258] in which inflammatory mediators (e.g., serotonin, bradykinin, prostaglandins [166,307,341]), chemical irritants (e.g., formalin [110]), or biological irritants (e.g., carrageenan, CFA) are injected in the paws to assess nociception and ongoing inflammatory pain. A CFA-soaked cuff can also be directly applied to the sciatic nerve to produce neuritis, a localized inflammation, that can also induce mechanical/thermal hypersensitivity (see [39,93–95]). Electrical stimulation is also sometimes used, but this form of stimulation does not accurately reflect naturally occurring threats for rodents [21]. Inflammatory pain is an equally important component in clinical pain conditions worthy of addressing [392], but I felt it necessary to limit the scope of my review to examples of mechanical and/or thermal hypersensitivity induced by neural injury.

Table 2. Non-exhaustive review of the behavioral evidence for pain-like states in rat models of surgically-induced neural injury. References (superscript corresponds to the table, number in brackets corresponds to bibliography): [1]Andrews et a., *Eur J Pain*, 2012 [11]; [2]Attal *et al*., *Pain*, 1990 [13]; [3]Baastrup *et al*., *Brain Res*, 2011 [15]; [4]Baastrup *et al*., *Pain*, 2010 [16]; [5]Baastrup *et al*., *Scand J Pain*, 2018 [14]; [6]Bannister *et al*., *Pain*, 2017 [19]; [7]Basbaum, *Exp Neurol*, 1974 [22]; [8]Bedi *et al*., *J Neurosci*, 2010 [28]; [9]Bennett and Xie, *Pain*, 1988 [31]; [10]Bravo *et al*., *Anesthesiology*, 2012 [42]; [11]Bravo *et al*., *Pain*, 2013 [41]; [12](Burke *et al*., *Brain Behav Immun*, 2014 [48]; [13]Burke *et al*., *Genes Brain Behav*, 2013 [47]; [14]Carlton *et al*., *Pain*, 2009 [57]; [15]Chen *et al*., *PLoS ONE*, 2014 [65]; [16]Clatworthy *et al*., *Neurosci Lett*, 1995 [77]; [17]Dalm *et al*., *Pain*, 2015 [85]; [18]Decosterd and Woolf, *Pain*, 2000 [88]; [19]Detloff *et al*., *Exp Neurol*, 2010 [90]; [20]Ding *et al*., *Behav Brain Res*, 2010 [96]; [21]Djouhri *et al*., *J Neurosci*, 2006 [105]; [22]Djouhri *et al*., *Pain*, 2012 [103]; [23]Dowdall *et al*., *Pharmacol Biochem Behav*, 2005 [108]; [24]Eschenfelder *et al*., *Pain*, 2000 [116]; [25]Fukuhara *et al*., *Cell Mol Neurobiol*, 2012 [125]; [26]Galan-Arriero *et al*., *Neurosci Lett*, 2015 [126]; [27]Goncalves *et al*., *Exp Neurol*, 2008 [134]; [28]Grace *et al*., *Brain Behav Immun*, 2018 [137]; [29]Grace *et al*., *Proc Natl Acad Sci U S A*, 2016 [138]; [30]Hogan *et al*., *Anesthesiology*, 2004 [165]; [31]Hu and Xing, *Pain*, 1998 [167]; [32]Huang *et al*., *Pain*, 2012 [169]; [33]Hubbard *et al*., *Neuroimage*, 2015 [170]; [34]Hulsebosch *et al*., *J Neurotrauma*, 2000 [171]; [35]Kim and Chung, *Pain*, 1992 [163]; [36]Kim *et al*., *Exp Brain Res*, 1997 [190]; [37]King *et al*., *Nat Neurosci*, 2009 [192]; [38]Kingery *et al*., *Pain*, 1993 [193]; [39]Kontinen *et al*., *Pain*, 1999 [198]; [40]Krupina *et al*., *Bull Exp Biol Med*, 2002 [201]; [41]LaGraize and Fuchs, *Exp Neurol*, 2007 [206]; [42]LaGraize *et al*., *Exp Neurol*, 2006 [205]; [43]Lee *et al*., *Eur J Pain*, 2003 [215]; [44]Lee *et al*., *Exp Brain Res*, 1998 [214]; [45]Lee *et al*., *Neuroreport*, 2000 [213]; [46]Leite-Almedia *et al*., *Pain*, 2012 [217]; [47]Li *et al*., *Mol Cell Neurosci*, 2003 [220]; [48]Liu *et al*., *Sci Rep*, 2015 [226]; [49]Luedtke *et al*., *J Neurotrauma*, 2014 [232]; [50]Ma *et al*., *Mol Pain*, 2010 [234]; [51]Maldonado-Bouchard *et al*., *Brain Behav Immun*, 2016 [236]; [52]Maves *et al*., *Pain*, 1993 [242]; [53]McNabb *et al*., *Neurosci Lett*, 2012 [243]; [54]Ning *et al*., *Neurol Res*, 2014 [261]; [55]Qu *et al*., *Biomed Res Int*, 2016 [293]; [56]Qu *et al*., *Pain*, 2011 [291]; [57]Roeska *et al*., *Pain*, 2009 [303]; [58]Sang *et al*., *Mol Pain*, 2018 [310]; [59]Seltzer *et al*., *Pain*, 1990 [319]; [60]Seminowicz *et al*., *Neuroimage*, 2009 [320]; [61]Shao *et al*., *Evid Based Complement Alternat Med*, 2015 [325]; [62]Sheth *et al*., *Pain*, 2002 [327]; [63]Song *et al*., *J Neurophysiol*, 1999 [333]; [64]Sweitzer *et al*., *J Pharmacol Exp Ther*, 2001 [339]; [65]Sweitzer *et al*., *Neuroscience*, 2001 [338]; [66]Tabo *et al*., *Pain*, 1999 [340]; [67]Tawfik *et al*., *J Pharmacol Exp Ther*, 2001 [345]; [68]Vierck *et al*., *J Pain*, 2005 [350]; [69]Wall *et al*., *Pain*, 1979 [354]; [70]Wang *et al*., *Anesthesiology*, 2011 [363]; [71]Wang *et al*., *BMC Neurosci*, 2015 [364]; [72]Wei *et al*., *Pharmacol Biochem Behav*, 2013 [366]; [73]Wu *et al*., *J Pain*, 2010 [375]; [74]Wu *et al*., *Pain*, 2013 [377]; [75]Xie *et al*., *Neural Plast*, 2016 [382]; [76]Yang *et al*., *J Neurosci*, 2014 [389];

[77]Zeltser *et al*., *Pain*, 2000 [395]; [78]Zeng *et al*., *Brain Res*, 2008 [397]; [79]Zhang *et al*., *J Neurophysiol*, 1999 [400]; [80]Zhang *et al*., *Neural Plast*, 2015 [401]; [81]Zhang *et al*., *Neurosci Lett*, 2008 [402]. CCI, chronic constriction injury; CCD, chronic compression of DRG; CPP, conditioned place preference; DRG, dorsal root ganglia; EPM, elevated plus-maze; EZM, elevated zero-maze; FST, forced swim test; L4/L5/L6, lumbar segment 4, 5, or 6; PSI, partial sciatic injury; SCI, spinal cord injury; SNI, spared nerve injury; SNL, spinal nerve ligation.

Model	Level of Injury	Sensory dysfunctions that suggest altered nociception	Motivational states and directed behaviors that suggest pain perception	Comorbidities that suggest altered emotional states	
				Anxiety-like behavior	Depression-like behavior
Contusive SCI	Spinal cord	Mechanical/thermal hypersensitivity [3, 4, 5, 8, 14, 19, 34, 51, 74, 76]	Evoked vocalization, Escape/avoidance, CPP[3, 4, 34, 51, 76]	Decreased exploration in open field, Decreased open-arm activity on EPM, Decreased burrowing[3, 5, 49, 51]	Anhedonia, Decreased social exploration, Decreased activity in FST[49, 51]
Rhizotomy	Proximal roots	Mechanical/thermal hypersensitivity [24, 47, 62, 66]	Limb autotomy[7]	-	-
CCD	DRG	Mechanical/thermal hypersensitivity [15, 20, 31, 32, 48, 50, 54, 55, 63, 75, 79, 80, 81]	Escape/avoidance, Spontaneous licking[48, 75, 79]	-	-
SNL and variants	L4/L5/L6 spinal nerves	Mechanical/thermal hypersensitivity [1, 6, 21, 22, 23, 30, 35, 36, 37, 41, 42, 43, 44, 53, 56, 64, 65, 67]	Limb autotomy, Spontaneous licking, Learned avoidance, CPP[6, 21, 22, 30, 35, 37, 41, 42, 53, 56, 73]	Decreased exploration in open field, Decreased open-arm activity on EPM/EZM, Decreased burrowing, Note: no effect in light/dark box[1, 12, 13, 39, 61]	Inability to swim normally in FST[39]
CCI	Sciatic nerve	Mechanical/thermal hypersensitivity [2, 9, 10, 11, 17, 23, 25, 28, 29, 36, 38, 44, 52, 57, 68, 78]	Evoked vocalization and licking, Limb guarding, Escape/avoidance, Note: no effect of CPP[2, 9, 10, 11, 16, 17, 52]	Decreased open-arm activity on EPM, Note: no effect in open field, on EZM, or on marble burying[11, 57, 78]	Conflicting results for FST[10, 25, 78]
PSI	Sciatic nerve	Mechanical/thermal hypersensitivity [1, 23, 36, 44, 57, 59, 71]	Evoked vocalization and licking, Spontaneous licking[59]	Decreased burrowing, Conflicting results for EPM[1, 57, 71]	-
Misc. nerve transection	Sciatic/distal nerves	Mechanical/thermal hypersensitivity [23]	Limb autotomy[40, 69, 77]	Insufficient data[40]	Anhedonia[40]
SNI	Distal nerves	Mechanical/thermal hypersensitivity [18, 23, 26, 27, 33, 45, 58, 60, 70, 72]	Escape/avoidance, CPP[26, 46, 72]	Conflicting results for open field and EPM[27, 33, 46, 58, 60]	Decreased activity in FST, Anhedonia[27, 70]

During my review, I noted any behavioral evidence that would suggest the potential for pain perception and/or anxiodepressive phenotypes. This includes altered motivational states (e.g., altered preferences, avoidance of noxious stimuli, seeking relief) and/or nocifensive behaviors (e.g., tending to an injured paw, licking) whose elicitation potentially requires cognition. I also limited my review to anxiety- and depression-like behaviors as they are two of the most common comorbidities associated with neuropathic pain. There is simply insufficient evidence to draw strong conclusions about pain perception and alterations in emotional states in the rhizotomy, CCD, and miscellaneous nerve transection models. As I mentioned, limb autotomy prevents proper behavioral investigations in the rhizotomy and miscellaneous nerve transection models, but others have argued occurrence of limb autotomy itself is sufficient evidence for a pain-like state as autotomy reflects rats' direct response to dysesthesias and pain [78]. Limb autotomy is also observed following SNL, but less frequently. Studies using the CCD model preferentially use reflex tests, possibly explaining the lack of stronger evidence. However, one study does demonstrate CCD rats exhibit a thermal preference for the 30°C side of a hot plate over 35°C side [382], which could be interpreted as a passive avoidance behavior. There is ample evidence of pain-like states in rats with SCI, SNL, CCI, PSI, or SNI. For example, evoked vocalizations have been described in rats with SCI, CCI, or PSI when a mechanical force is applied to a sensitive region of the body above or below the site of SCI or affected paws in the case of CCI and PSI. It should be noted SCI-induced spasticity can be difficult to assess and it is possible reflexive responses evoked below injury are not cerebrally mediated [16]. Other nocifensive behaviors like spontaneous licking and limb guarding occur in rats with SNL, CCI, or PSI. During my review I did not encounter SNI studies with similar descriptions.

Much of the evidence for pain perception in **Table 2** comes from behavioral tests with more descriptive power than standard reflex tests; many of the studies were conducted during/after commentary critical of the pain field [253,255,351]. Operant tests like the PEAP

and CPP offer more conclusive evidence of pain perception [256,286,352]. Rats with SCI, SNL, CCI, or SNI will avoid mechanical stimulation (sometimes noxious, see [375]). This demonstrates the mechanical stimuli are considered to be aversive. Rats with SCI, SNL, or SNI exhibit a preference for analgesic-paired chambers, suggesting the presence of spontaneous pain and drive to seek relief. The CCI model appears to be an exception, Dalm *et al*. [85] report CCI rats do not develop CPP when bupivacaine is used for chamber pairing. They also do not see significant increases in dorsal horn neuronal activity that would suggest peripherally-driven spontaneous pain [85]. No other studies have investigated CCI-induced spontaneous pain using the CPP test.

The evidence for anxiety phenotypes in rats with SNL, CCI, PSI, or SNI are inconsistent, possibly due to multiple uncontrolled factors (e.g., missing controls, low power; see [29,253,290,334]). For example, Bravo *et al*. [41] do not report naïve controls for the marble burying test, which might have revealed both sham and CCI rats develop anxiety. Inconsistencies across studies might also reflect heterogeneity in rats and humans in regards to development of pain-associated comorbidities. One study shows PSI decreases spontaneous burrowing behavior [11], but it is unclear whether it is due to evoked pain in affected limbs or ongoing/spontaneous pain, or if burrowing is a general measure of an animal's well-being [87,177]. There is even less evidence from which to draw strong conclusions about injury-induced depression. Unintended damage to the L4 nerve during the SNL surgical procedure can cause partial paralysis (see [163]), which might explain for rats' inability to swim in the Kontinen *et al*. study [198]. Otherwise, the presence of anhedonia (i.e., inability to feel pleasure; injured rodents do not exhibit a preference for sucrose-flavored water) in some of the models does accurately reflect human conditions, especially in persons with SCI [371]. Greater emphasis needs to be placed on using behavioral tests that might reveal anxiety, depression, and other comorbidities associated with neuropathic pain.

1.4. Using an operant mechanical conflict test to reveal pain-avoidance behaviors in freely behaving rodents

Some of the strongest evidence for pain caused by neural injury comes from operant behavioral tests. One of the tests for nociception/pain perception I included in **Table 1**, but did not discuss in context of **Table 2**, is the operant MC test and mechanical conflict system (MCS) device developed by Harte *et al*. [155] (see also [210]). The MC test is theoretically a marked improvement over mechanical reflex tests and operant tests like the PEAP which capture rodents' decisions to avoid noxious stimulation. I think the MC test and device might be appealing to pain researchers due possible improvements in descriptive power of pain-avoidance behaviors. Unfortunately, it is a relatively new test and has received little attention compared to many other tests I described. In this section I want to briefly cover the benefits associated with the MC test and review current literature. This will set the stage for my behavioral study in *Chapter* 2.

The MC test is an operant behavioral paradigm that combines "dose"-dependent testing of evoked noxious stimuli with free-choice; rodents can decide whether or not they want to escape from a brightly lit chamber by crossing over a floor lined with sharp probes. Rodents assess the painful risk associated with crossing the probes and exhibit longer latencies to escape the brightly lit chamber as probe height increases [155]. The currently established outcome measure is the escape latency (i.e., time it takes a rodent to leave the light chamber and step onto the probes with all 4 paws). Under painful neuropathic conditions like CCI [155], diabetic neuropathy [143], and SNI [326], rats and mice exhibit longer escape latencies. Among other benefits, the test removes the potential for unconscious experimenter bias inherent in reflex tests and PEAP that utilize hand-held stimuli [38]. The fact that freely behaving rodents actively avoid the probes demonstrates they find them to be truly aversive, thus providing stronger evidence for pain-like states in the aforementioned conditions. Brain regions that

mediate the affective-motivational components of pain are recruited during performance in the MC test [271], similar to the PEAP [205,206,403].

1.4.1. Review of current literature using the operant mechanical conflict test

There are currently only 6 studies that report use of the MC test and device. Careful review (see **Table 3** and references in legend) shows there is an underrepresentation of critical sham controls in studies that report use of injury models, and the majority of studies rely upon a single behavioral metric to describe pain-avoidance behavior. Reflex tests are also omitted for validation of mechanical nociception in several studies. The earliest known study from 2012 measures the time rats spend on the probes [210]. Rats with a blunt force SCI injected with a herpes simplex virus-based gene transfer vector for the anti-inflammatory cytokine interleukin-10 (vIL10) spend more time on the probes than SCI rats injected with control vector. Lau *et al*. conclude SCI-induced pain and performance in the MC test are influenced by activation of the neuroimmune system. One would reasonably extrapolate that naïve and/or sham rats behave similarly to vIL10-treated SCI rats, but these controls were not introduced to the MC test even though the study mentions use of shams for other experiments. No other study has followed up on measuring time on the probes. In 2016, Harte *et al*. describe a more rigorous training regimen and reproducible outcome measure to be used for the MC test (i.e., escape latency) [155]. Rats with CCI exhibit longer escape latencies than naïve controls on probes ranging 0.5 to 4 mm in height. They also demonstrate analgesics like pregabalin and morphine attenuate this behavior. Again, sham rats were not tested in any context. Later studies investigating painful diabetic neuropathy in rats [143], morphine dependency in naïve rats [271], and SNI in mice [326] all measure escape latency without other measures of performance in the MC test. Sham surgery controls were not relevant in the diabetic neuropathy or morphine dependency study. The SNI study is particularly interesting for several reasons: 1) it is the only study to use mice, 2) it includes use of sham surgery controls, and 3) it includes use of the opioid analgesic buprenorphine on both SNI and sham mice at 8 days post-surgery. Shepherd and Mohapatra

demonstrate that SNI in mice, like CCI in rats [155], increases escape latency and is attenuated by buprenorphine [326]. However, the experiment appears to be missing a critical vehicle-treated sham group that is not mentioned in the study. At 8 days post-surgery buprenorphine may have masked acute postsurgical pain in sham mice, but this possibility cannot be addressed without a vehicle-treated sham group for comparison. It is not explained why this control was not performed. Furthermore, the SNI effect on escape latency is skewed by 3 of the 8 mice tested. There appears to be a bimodal distribution in escape latencies, which suggests escape latency is not effectively capturing some aspect of pain-avoidance behavior. Perhaps the underlying pain-like state induced by SNI is not uniform in rodents – like other peripheral neuropathies in humans [20] – and can be qualitatively/quantitatively distinguished. Indeed, other studies will sometimes segregate rodents (e.g., no “pain”, yes “pain”) based on measures of other behavioral tests. The most recent study (2018) using the MC test explores the role of exercise therapy in mitigating neuropathic pain following contusive SCI in rats [67]. Chhaya *et al*. segregate SCI rats into “pain” and “no pain” groups based on their percent change from baseline in mechanical withdrawal thresholds following SCI. The authors conclude that exercise promotes a modest improvement in the MC test (i.e., reduced escape latency), but the effects are not statistically significant. Unfortunately, no sham control groups were included for comparison. Chhaya *et al*. also demonstrate that escape latencies in SCI rats with or without exercise, and with or without pain, do not correlate with von Frey reflex tests. This suggests the two tests may not reflect similar aspects of pain-related behavior.

Table 3. Summary of research articles that report use of the operant mechanical conflict test. References (superscript corresponds to the table, number in brackets corresponds to bibliography): [1]Lau *et al*., *Neurorehabil Neural Repair*, 2012 [210]; [2]Harte *et al*., *PLoS ONE*, 2016 [155]; [3]Griggs *et al*., *J Pain*, 2016 [143]; [4]Pahng *et al*., *Neuroscience*, 2017 [271]; [5]Shepherd and Mohapatra, *Neuropharmacology*, 2018 [326]; [6]Chhayah *et al*., *J Neuroatrauma*, 2018, in press [67]. CCI, chronic constriction injury; CFA, complete Freund's adjuvant; IL-10, cytokine interleukin-10; NSAID, nonsteroidal anti-inflammatory drug; PDN, peripheral diabetic neuropathy; SCI, spinal cord injury; SNI, spared nerve injury; ZDF, Zucker Diabetic Fatty rats; ZL, Zucker Lean rats.

Model/Reference	Species	Test methodology				Results	Critiques		
		Test groups	Training (no probes)	Probe Height (mm)	Outcome measure		Shams used in study?	Shams used in operant MC test?	Other reflex tests with shams?
Contusive SCI (T12-L1)[1]	Rat	SCI w/ IL-10 vector, SCI w/ control vector	3 days	2 and 3	Time on probes (s)	Treating SCI with an IL-10 vector increases time on probes	Yes	No	Yes; mechanical hyperalgesia, mechanical allodynia, thermal hyperalgesia
SNI (spared sural)[5]	Mouse	Sham w/ opioid, SNI w/ saline, SNI w/ opioid	Authors refer to Harte et al., 2016 paper	0, 2, and 5	Escape latency (s)	SNI increases escape latency, buprenorphine reverses SNI effect	Yes	Yes, but only with buprenorphine	No
CCI[2]	Rat	CCI, naïve	Familiarization = 1-2 days; Training = 3-5 days	0 to 4	Escape latency (s)	CCI increases escape latency	No	-	-
Hemi-contusive SCI (C5)[6]	Rat	SCI w/ exercise, SCI w/o exercise	Training = 3 days	Not listed	Escape latency (s)	Exercise therapy does not reduce escape latency	No	-	-
Type 2 PDN[3]	Rat	ZDF, ZL	Familiarization = 1 day; Training = 4 days	0, 1, 3, and 4	Escape latency (s)	Type 2 PDN increases escape latency	n/a	-	-
Morphine-dependency[4]	Rat	Chronic morphine, chronic saline	Familiarization = 1-2 days; Training = 3 days	0 to 5	Escape latency (s)	Morphine-dependency increases escape latency	n/a	-	-
CFA[5]	Mouse	Saline w/ NSAID, CFA w/ saline, CFA w/ NSAID	Authors refer to Harte et al., 2016 paper	0, 2, and 5	Escape latency (s)	CFA increases escape latency, carprofen reverses CFA effect	n/a	-	-

1.4.2. Improving the validity of the operant mechanical conflict test

Several studies have demonstrated the MC test to be useful for assessing mechanical nociception and pain-avoidance behavior. However, a thorough review of current literature has revealed missing information and several unanswered questions. I think there is an unmet need for additional validation of multiple neuropathic injury models with their appropriate sham control groups and more thorough quantitative analyses to better understand how neuropathic pain conditions influence avoidance behaviors in the MC test. Experiments that address the following questions will improve the validity of the MC test as an informative test for revealing aversive pain-like states. To outline:

1) Do rodents in sham-operated control groups for surgically-induced neuropathic pain conditions also avoid the probes in the MC test?
2) Are there additional behaviors elicited during the MC test that could more effectively reveal the presence of an aversive pain-like state?
3) Does the standard reflex measure of mechanical sensitivity accurately predict pain-avoidance behavior in the MC test?

Chapter 2: *Persistent postsurgical pain caused by sham surgeries for neuropathic pain models is revealed by behavioral alterations in an operant conflict test*

Disclosure: The work described in this chapter was performed in collaboration with the Grace Lab at The University of Texas MD Anderson Cancer Center in Houston, TX, that includes the following: Peter M. Grace, PhD, Michael J. Lacagnina, PhD, and Jiahe Li, PhD. The Grace Lab has given their permission for portions of the text, results, and figures relating to the use rats with a chronic constriction injury, and related controls, to be included in this chapter. A manuscript has been submitted to the journal *Pain* with Max A. Odem, Michael J. Lacagnina, and Stephen L. Katzen as co-first authors. Additional authors include Jiahe Li, Peter M. Grace, and Edgar T. Walters as the corresponding, senior author.

2.1. Rationale

In principle, an operant test in which an animal's voluntary behavior discloses the aversiveness of a test stimulus might reveal evoked pain that has not been evident in reflex tests. We modified the MC test to take advantage of rats' innate drive to explore novel environments [113], allowing efficient measurement of pain-related changes in a rat's motivation to repeatedly cross noxious probes. Prior studies using the MC test usually habituated exploratory behavior prior to testing and permitted only a single crossing of the probes, measuring the escape latency [67,143,155,210,271,326]. Pilot experiments using sham-operated and rats with SCI tested several months post-surgery revealed both groups avoided the noxious probes, suggesting the MC test may reveal persistent postsurgical alterations in behavior. Humans often experience painful hypersensitivity long after surgical procedures similar to those used to expose peripheral nerves or the spinal cord in rodent neuropathic pain models [64]. Sham controls are sorely underrepresented in prior studies that use the MC test [67,143,155,210,326]. Here we use our modified MC test to reveal previously

unrecognized postsurgical alterations in behavior after the sham surgeries for a thoracic T10 SCI, L5 SNT, and CCI of the sciatic nerve.

2.2. Materials and methods

2.2.1. Animals

All procedures followed the guidelines of the International Association for the Study of Pain and were approved by the Animal Care and Use Committees for the University of Texas Health Science Center at Houston (UTHealth) and the University of Texas MD Anderson Cancer Center. Male, Sprague-Dawley rats (Envigo, USA) were used at both institutions. At McGovern Medical School, the rats (250-300 g, 2 per cage) were acclimated to a controlled environment (12-hour reverse light/dark cycle, 21 ± 1°C) for ≥4 days before beginning experiments. The corn cob bedding was replaced 2-3 times per week while food and water were provided *ad libitum*. At MD Anderson Cancer Center, the rats (10 weeks old, 2-3 per cage) were acclimated to the controlled laboratory environment (12-hour light/dark cycle, lights on at 07:00 h, 22 ± 1°C) for at least 7 days before beginning experiments. The corn cob bedding was replaced once per week while food and water were provided *ad libitum*.

2.2.2. Injury models and surgical procedures

Spinal cord injury

Surgeries were performed at McGovern Medical School as previously described [25,28,377,389]. Anesthesia in most of the studies (see **Figures 4-6**) was by isoflurane (induction 4-5%; maintenance 1-2%). In the remainder (see **Figure 3** and **7**), intraperitoneal (i.p.) injection of ketamine (60 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg) was used. Rats were determined to be deeply anesthetized and areflexic before proceeding. Local anesthetic (bupivacaine, 2 mg/kg) was delivered subcutaneously (s.c.) at the incision site near T10 before incising the skin from T8-T12. Laminectomy of the T10 vertebrae was followed by

contusion impact (150 kdyne, 1 s dwell time) using an Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation, LLC, Fairfax Station, VA, USA). Following impact, the paravertebral muscles were closed with vicryl-coated, absorbable suture and the skin incision was closed with 9 mm wound clips. Sham rats received the same laminectomy surgery minus spinal impact. Rats were returned to their home cage and placed on a heating pad maintained at 37°C. The analgesic buprenorphine hydrochloride (0.02 mg/kg; Buprenex, Reckitt Benckiser Healthcare Ltd., Hull, England, UK) was administered in 0.9% saline (2 mL/kg, i.p.) twice, daily up to 5 days post-surgery. The prophylactic antibiotic enrofloxacin (0.3 mL; Enroflox, Norbrook, Inc., Overland Park, KS, USA), was also administered in 0.9% saline daily up to 10 days post-surgery. Manual bladder evacuations were performed twice, daily until rats recovered neurogenic bladder voiding. The day after surgery hindlimb locomotion was assessed using the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale [24]. Only sham rats with BBB scores of 21 for both hindlimbs were accepted. The majority of SCI rats were scored a 0 or 1 for both hindlimbs 1 day after surgery. Naïve rats were transported to the surgical suite at the same time as surgeries were performed, but were otherwise left undisturbed in their home cages.

Spinal nerve transection

Surgeries were performed at McGovern Medical School. A modified version of the SNL model [163] was used in which the L5 spinal nerve was transected without ligation [338,339,345], herein referred to as the SNT procedure. Rats were anesthetized using isoflurane (induction 4-5%; maintenance 1-2%) and local anesthetic (bupivacaine, 2 mg/kg, s.c.) was used before incising the skin above the lumbar spine. The left transverse process at L6 was removed and the ventral rami of the L4 and L5 spinal nerves were exposed. The L5 nerve was axotomized using microdissection scissors and a 1-2 mm segment of the distal L5 stump was removed. Manipulation of the L4 nerve was minimal, it was not cut. The subcutaneous layers were sutured closed using vicryl-coated absorbable suture and the

cutaneous layer was closed using 9 mm wound clips. The sham surgery was the same minus transection of the L5 nerve. An analgesic (0.02 mg/kg; Buprenex) was administered twice, daily (2 mL/kg, i.p.) up to 2 days post-surgery and an antibiotic (0.3 mL; Enroflox) was also administered daily up to 10 days post-surgery. Naïve rats were transported to the surgical suite, but were otherwise left undisturbed in their home cages.

Chronic constriction injury

Surgeries were performed at MD Anderson Cancer Center. Neuropathic pain from peripheral injury was induced using the CCI model of unilateral sciatic nerve injury [31] as previously described [137,138]. Rats receiving CCI or sham surgeries were anesthetized with isoflurane (4% in oxygen for induction; 2-3% maintenance) and placed on an electric heating pad. Skin at the mid-thigh level of the left leg was shaved with an electric razor and cleansed with povidone-iodine and 70% ethanol. An incision of the skin was made with a scalpel blade and the sciatic nerve was exposed through blunt dissection of the biceps femoris muscle. Using glass nerve hooks, a segment of the sciatic nerve was gently liberated from the surrounding connective tissue. For CCI surgeries, 4 ligatures (4-0 chromic gut; Ethicon, USA) were loosely tied around the sciatic nerve approximately 1 mm apart. For sham surgeries, the sciatic nerve was manipulated with nerve hooks and isolated in an identical fashion, but no chromic gut ligations were sutured around the nerve. The muscle layer was closed with non-absorbable sutures (4-0 silk; Ethicon, USA), 9 mm wound clips were applied to close the skin, and rats were then returned to their home cage and monitored post-operatively until fully ambulatory. Naïve rats were transported to the surgical suite, but were otherwise left undisturbed in their home cages.

2.2.3. Behavioral testing procedures

Habituation to ambient testing conditions

At McGovern Medical School, rats were acclimated to the behavioral testing room each morning for 1 hour under red light and constant background white noise generated by a TaskMasking speaker (K.R. Moeller Associates Ltd., Burlington, Ontario, Canada). Several days prior to testing the rats were acclimated to the presence of an investigator and the acrylic chambers (IITC Life Science Inc., Woodland Hills, CA, USA) used to isolate rats for hindpaw reflex tests. During gentling (i.e., handling and acclimation to experimenters) the rats were placed in the chambers on raised wire-mesh platforms for 20 minutes and periodically fed sweetened cereal. Two experimenters were female (<30 years old) and one experimenter was male (>30 years of age). Male and female experimenters did not perform tests on the same days in order to limit male-induced stress and analgesia [334].

At MD Anderson Cancer Center, rats were acclimated to the behavioral testing room for at least 1 hour under red light illumination prior to each behavioral test. Rats were handled by the experimenter in 5 min sessions over 3 days. Habituation to hindpaw reflex testing occurred by placing rats in acrylic chambers on raised wire-mesh platforms for 60 min sessions over 3 days. One female and one male experimenter (>30 years of age) performed all experiments, and each rat was always manipulated by the same experimenter.

Hindpaw mechanical sensitivity

At McGovern Medical School, hindpaw sensitivity to mechanical stimuli was measured at 1-2 months post-surgery for naïve, sham, and SCI rats. For SNT experiments, naïve, sham, and SNT rats were tested up to 1.5 weeks post-surgery. Following habituation and gentling procedures, the rats were placed in acrylic chambers and the 50% mechanical withdrawal threshold was assessed using the “up-down” method [63,90,98,99] of presenting calibrated von

Frey filaments (Stoelting Co., Wood Dale, IL, USA). The range of logarithmically incremental filaments used included (in grams): 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, 26.0, 60.0. Filaments (starting with 6.0 g) were presented perpendicularly to the plantar surface of the hindpaw between the footpads at a constant speed until the filament bent. Filaments were held for ~1 s before removal. Each hindpaw was presented with a series of 10 stimuli, spaced 30 s apart to provide consistent testing durations and treatment. A rapid, robust withdrawal of the hindpaw from the filament was considered to be a positive response and care was taken to not present stimuli during ambulatory movements in the chamber. In experiments using SCI rats the withdrawal thresholds for the left and right hindpaws were calculated separately and then averaged together for a single score per rat. Thresholds for the ipsilateral (side of injury) and contralateral hindpaws were calculated separately for experiments with SNT and sham rats. For CCI experiments at MD Anderson Cancer Center, the naïve, sham, and CCI rats were tested 2 weeks post-surgery using the "up-down" method. The range of filaments included (in grams): 0.4, 0.6, 1.2, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0. Filaments were administered to the distal portion of the heel [137,138]. The mechanical thresholds for the two hindpaws were scored separately.

All mechanical withdrawal thresholds were log transformed to account for Weber's Law [251]. Mills *et al*. demonstrate that the original equation used to calculate the 50% paw withdrawal threshold (PWT; in grams) described by Chaplan *et al*. [63] can be reduced to the following:

$$\text{Log}(\text{PWT}) = Xf + K\delta - 4$$

where Xf = the final filament used (i.e., the filament handle #), K = the tabular value for the delivered sequence of test stimuli (refer to table in [63]), and δ = the mean difference between the delivered sequence of test stimuli (calculated using the filament handle #'s). The filament handle # = Log_{10} of (10 x filament force in milligrams) (Stoelting Co. Touch Test™ Sensory

Evaluators Operation Manual), indicating the handle #'s can be used in the Chaplan or Mills equations without converting gram forces into log units. Also, δ is not a fixed value. Note that gram forces between 1 g and 0 yield negative numbers when log transformed.

Hindpaw heat sensitivity

Hindpaw sensitivity to radiant heat [152] was measured at 1-2 months post-surgery for naïve, sham, and SCI rats. Experiments with SNT and CCI rats did not test heat sensitivity in order to limit the exposure to hyperalgesic test stimuli that might influence the results of the subsequent MC tests. Once habituated and gentled, the rats were placed in acrylic chambers on a glass platform (Plantar Analgesia Meter; IITC Life Science Inc., Woodland Hills, CA, USA) and acclimated to the 30°C temperature-controlled surface [97] for 20 minutes. Settings for the radiant heat stimulus: idle beam intensity = 10%, active beam intensity = 45%, active beam cutoff = 20 seconds. While idle, the light beam was positioned between the footpads of either the left or right hindpaw, and once positioned the active beam was turned on. A rapid, robust withdrawal of the hindpaw was considered a positive response to the radiant heat stimulus. Rats that did not exhibit a withdrawal by the time of the automatic active beam cutoff were given a score of 20 seconds. Tests continued until the withdrawal latency was recorded 5 times for each hindpaw, switching back and forth between the hindpaws every 30 seconds. If ambulatory movements occurred during presentation of the stimulus the active light beam was turned off, the experimenter waited 30 seconds, and the other hindpaw was tested. The average withdrawal latency for each hindpaw was calculated using the 3 middle latencies, the highest and lowest latencies were omitted. The two hindpaw latencies were then averaged together for a single score per rat.

Operant mechanical conflict tests

Voluntary pain-related aversion to a noxious stimulus was assessed using the mechanical conflict system (MCS; Coy Laboratory Products, Inc., Grass Lake, MI, USA). The

MC test presents rats with a choice in responding to two aversive stimuli – remain exposed to an aversive bright light or escape the light by crossing a floor covered with sharp probes. Longer latencies to leave the light chamber indicate increased motivation to avoid the probes, and escape latency is currently the most common measure of pain-related behavior in the MC test [67,143,155,271,326]. We found (pilot studies and see **Figure 3**) that when the MCS is still relatively novel, uninjured rats cross the noxious probes multiple times. The repeated return to the brightly lit chamber across the sharp probes indicates the presence of a second motivation to cross the probes, which is probably the rats' exploratory drive in a novel environment [113]. We modified the MC paradigm of Harte *et al*. [155] so that both motivations to cross – 1) to escape the light and 2) to explore the MCS – were in conflict with the aversiveness of the noxious probes.

Harte *et al*. describe a lengthy familiarization procedure to the MCS that lasts 1-2 days followed by an escape training procedure that lasts 3-5 days, with a total of 10 to 19 opportunities (each 5 minutes duration) for the rat to explore the MCS before experiencing the sharp probes [155]. During this training the rats learn that when the exit door opens they can escape from the light room and reach the dark room. We abbreviated the MC test by combining the familiarization and training procedures into three 5 minute familiarization trials without the probes, repeated 3 times on day 1, spaced 30-60 minutes apart. In each trial: 1) a rat was placed inside the light chamber with the lid closed, the light off, and the exit door closed, 2) after 20 seconds the light was turned on, 3) after 15 seconds the exit door was opened when (or if) the rat faced the exit, 4) the rat freely explored all 3 chambers in the MCS for 5 minutes, 5) the was rat was returned to its home cage, and 6) the MCS was thoroughly cleaned with 70% ethanol (in distilled water) in preparation for the next trial. The rats rapidly learned to escape the light room as soon as the exit door was opened. Indeed, rats sometimes attempted to lift the door on their own by the second or third trials. For SNT experiments, rats received a

second day of 3 trials without probes the 1 day after surgery to assess any acute postsurgical effects on movement in the absence of probes before later testing with the probes.

After the familiarization trials, rats underwent a 1-day testing sequence in which they were challenged with the probes. The first trial (baseline) was without probes to reacquaint the rats with the MCS. In the first study (**Fig. 2**, first SCI timeline), probe height was successively increased to 1, 2, 3, and 4 mm in 3-minutes trials spaced ~30 minutes apart. Probe heights were presented in ascending order to minimize possible sensitizing effects from higher probes and to permit testing multiple probe heights per rat on a single day. A shorter, 3-trial protocol in which rats were challenged with the probes twice was used in subsequent experiments (**Fig. 2**, second SCI, SNT, and CCI timelines). The testing sequence started with a single trial at 0 mm, followed by 2 trials at 4 mm, 5 minutes per trial, spaced ~30 minutes apart. All SCI rats were capable of weight-supported plantar stepping with BBB scores ≥10 by the time of testing, meaning they could readily traverse the probes without bodily harm.

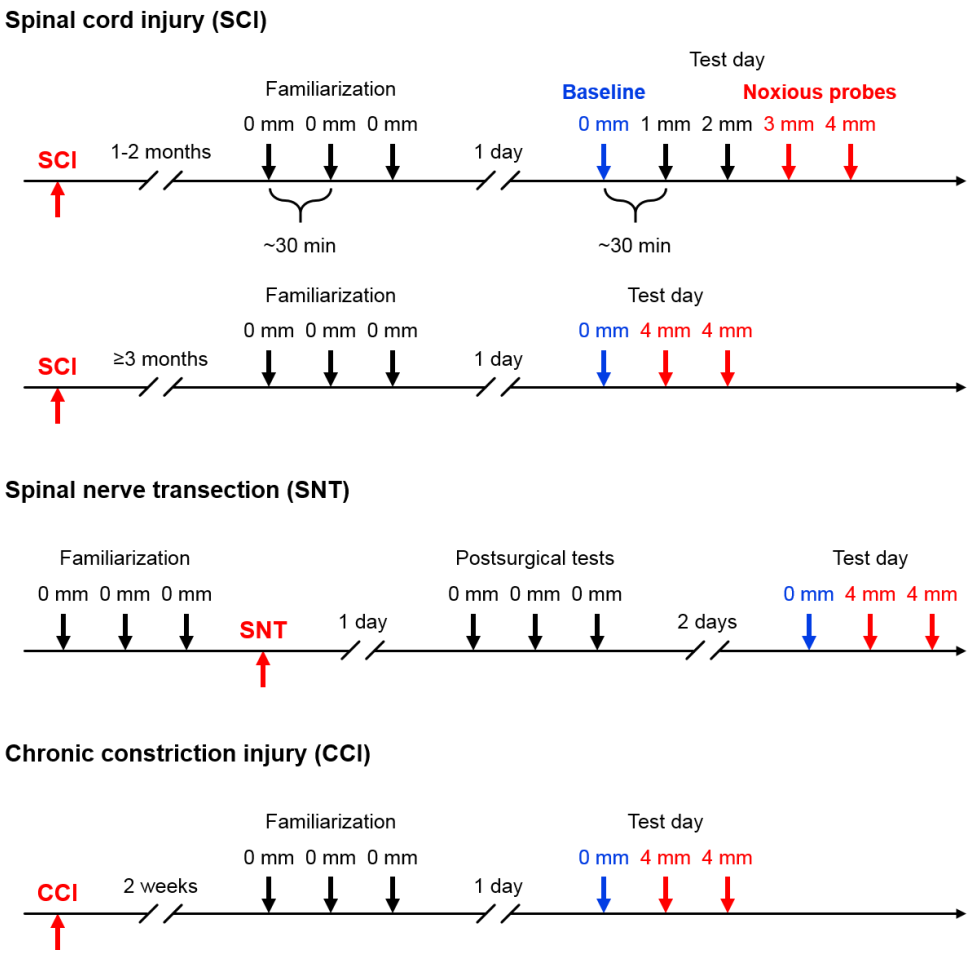


Figure 2. Timelines of the operant mechanical conflict test used to measure changes in avoidance of noxious probes in three neuropathic pain models and their sham-surgery controls. Numbers (in mm) indicate elevation of the sharp probes above the floor of the middle chamber. Familiarization refers to the 5-minute periods in which the rat is free to explore the 3-chamber test device in the absence of elevated probes (0 mm). On the probe exposure day (test day), a baseline exposure to the 0-mm probe condition is given for comparison to responses during the two subsequent noxious probe exposures. CCI, chronic constriction injury; mm, millimeter; SCI, spinal cord injury, SNT, spinal nerve transection. Figure prepared by Max A. Odem.

All trials during training and probe testing were video recorded in 1080i resolution at 30 frames per second using a Panasonic HC-V750 camcorder (Panasonic Corporation, Osaka, Japan) or 1080p resolution at 30 frames per second using an Apple iPhone (Apple Inc., Cupertino, CA, USA) and scored by a blinded experimenter. The following measures were

collected for post-hoc analysis: 1) the escape latency during the first crossing, 2) the number of crossings of the probe chamber, and 3) the total time elapsed to the completion of the second crossing. A subset of videos were scored for behavioral measures that might reveal above-level mechanical hypersensitivity in the forepaws [28]. The number of times each rat withdrew one of their forepaws after contacting the probes was scored. No formal definitions of a crossing currently exist for the MC test, and measures other than escape latency depend upon this definition. We defined the first crossing as the rat placing all 4 paws inside the dark chamber after leaving the light chamber. Every subsequent crossing was defined as the rat placing its head and two forepaws inside the light or dark chamber.

2.2.4. Data analysis and experimental design

Statistical analyses were performed using Prism v7.03 (Graphpad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error (SEM), median with interquartile range, or as incidence (% of rats tested). The Shapiro-Wilk test was used to assess normality for continuous measures. Planned comparisons between naïve, sham, and injury groups were made using 1-way ANOVA or Kruskal-Wallis tests followed by Tukey's or Dunn's post-hoc tests. Comparisons between incidence measures were made using Fisher's exact tests with Bonferroni corrections for multiple comparisons. Significance for all statistical tests was set at $P < 0.05$ and all reported P values are two-tailed.

Hindpaw withdrawal measures

Mechanical withdrawal thresholds collected from naïve, sham, and SCI rats at 1-2 months post-surgery by a single female experimenter were compared using a 1-way ANOVA followed by Tukey's post-hoc test. Potential relationships between mechanical withdrawal thresholds and measures of SCI severity (contusion displacements and day 1 post-SCI BBB scores) were assessed by a Spearman correlation. Heat withdrawal latencies collected from naïve, sham, and SCI rats at 1-2 months post-surgery by a single female experimenter were

compared using 1-way ANOVA followed by the Tukey's post-hoc test. For SNT and CCI experiments, mechanical withdrawal thresholds for the ipsilateral (injured) and contralateral (uninjured) paws were compared, separately, across naïve, sham, and injured groups using a 1-way ANOVA or Kruskal-Wallis test followed by Tukey's or Dunn's post-hoc test.

Operant mechanical conflict measures

To determine the effects of probe height on the number of crossings, 1, 2, 3, and 4 mm probes trials were compared to the baseline 0 mm probe trial on the same day using repeated measures 1-way ANOVA or Friedman tests followed by Dunnett's or Dunn's post-hoc test. Sphericity was not assumed when using the repeated measures 1-way ANOVA and the Geisser-Greenhouse correction was applied. Planned comparisons between groups were performed for trials with probe heights found to significantly reduce crossings. Subsequent SCI, SNT, and CCI experiments were performed with just two probe exposures using the 4 mm probes. Planned comparisons between groups were performed for both exposures. For SNT experiments, to determine whether acute postsurgical pain impacted crossings, additional baseline trials (3 total) without probes were performed 1 day following surgery and averaged together. Postsurgical trials (days 1 and 3) were compared to the averaged pre-surgery familiarization trials using the Friedman test followed by Dunn's post-hoc test. The CCI experiments were performed 2 weeks after surgery.

2.3. Results

2.3.1. Reduction of repeated voluntary crossing of noxious probes indicates that both SCI and sham surgery cause pain-related suppression of exploratory behavior

During the 3 familiarization trials without probes in the MCS, all rats in the naïve, sham, and SCI groups learned to exit the light chamber quickly (**Fig. 3A**). No significant differences between groups on the 3rd trial were found (1-way ANOVA P = 0.10). Escape latencies

measured 24 hours later during the single baseline trial without probes were almost unchanged (naïve: 13.0 ± 6.8 s, n = 8 rats; sham: 4.3 ± 1.9 s, n = 8 rats; SCI: 7.1 ± 2.7 s, n = 11 rats; 1-way Kruskal-Wallis $P = 0.50$). These are similar to but slightly longer than the escape latencies reported for rats that underwent more extensive familiarization and escape training [155]. Unexpectedly, many rats voluntarily crossed back into the light chamber as they explored the MCS during each familiarization trial (**Fig. 3B**). The mean number of crossings between groups during familiarization trial 3 were not significantly different (1-way ANOVA $P = 0.78$). Multiple returns to the light chamber occurred during familiarization trials in all experiments (SCI, SNT, and CCI; see below), suggesting that the motivation to continue exploring the MCS remained high enough to offset the aversiveness of the bright light, even after 3 exposures to the MCS. This raised two questions: 1) how would exploratory behavior (as indicated by multiple crossings) be affected by noxious probes in the middle chamber, and 2) would the response to the probes be altered by prior neural injury or surgical injury?

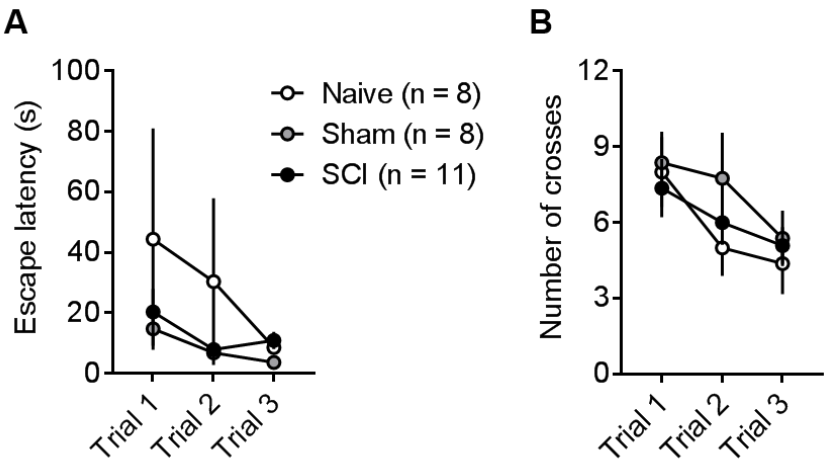


Figure 3. Rats make multiple crossings in the brightly lit mechanical conflict system when noxious probes are not present. The abbreviated familiarization and training procedure consisted of three 5-minute trials spaced ~30 minutes apart. Rats were tested at ~3 months post-surgery. Escape latencies **(A)** and crossings **(B)** decreased as the groups habituated to the MCS. No significant differences between groups were found during trial 3, comparisons between groups were assessed using a 1-way ANOVA (escape latencies $P = 0.10$; crosses $P = 0.78$). Note that on average rats crossed back into the light chamber multiple times after escaping from the light chamber. Data shown as mean ± SEM. MCS, mechanical conflict system; SCI, spinal cord injury. **Contributions:** Max A. Odem designed experiments, analyzed data, and prepared figures; experiments were performed by Tamara McGhee, Kendra C. Wicks, and Emily A. Spence.

These questions were addressed by exposing a new cohort of naïve, sham-operated, and SCI rats to a series of trials (3 minutes each) with progressively ascending probe heights (0 to 4 mm), spaced 30 minutes apart (see **Figure 2**, first SCI timeline). The SCI and sham rats, but not the naïve rats, crossed the 3 and/or 4 mm probes significantly fewer times than they had crossed the middle chamber during the 0 mm baseline trial 90 to 120 minutes earlier (**Fig. 4A1** and **4A2**). There was a trend for SCI and sham rats to cross the 1 and 2 mm probes less than the naïve rats, but post-hoc comparisons did not reveal significant differences (1-way ANOVA $P = 0.14$ and 0.19 for 1 and 2 mm probes, respectively). The effects of the 3 and 4 mm

probes on escape latency during the first crossing were less clear (**Figs. 4B**). Some SCI and sham rats showed much longer latencies, and a few refused to cross the probes even once. At the same time, ~50% of the sham and SCI rats had latencies that were comparable to naïve rats. No rats were excluded based on deviant latencies to cross the probes. SCI rats were less likely to cross the probes multiple times than naïve rats, and sham rats showed a very similar trend (**Fig. 4C**), with ~50% of the SCI and sham rats refusing to cross the 3 and 4 mm probes a second time. These results indicate that 1) exploratory behavior (multiple crossings) in naïve rats shows little or no reduction by the presence of noxious probes in the middle chamber, 2) prior SCI increases avoidance of the probes (reduces crossing), and 3) the surgical injury used as a sham control for the SCI procedure increases avoidance of the noxious probes (1 months or longer after injury) similar to the reduction caused by SCI.

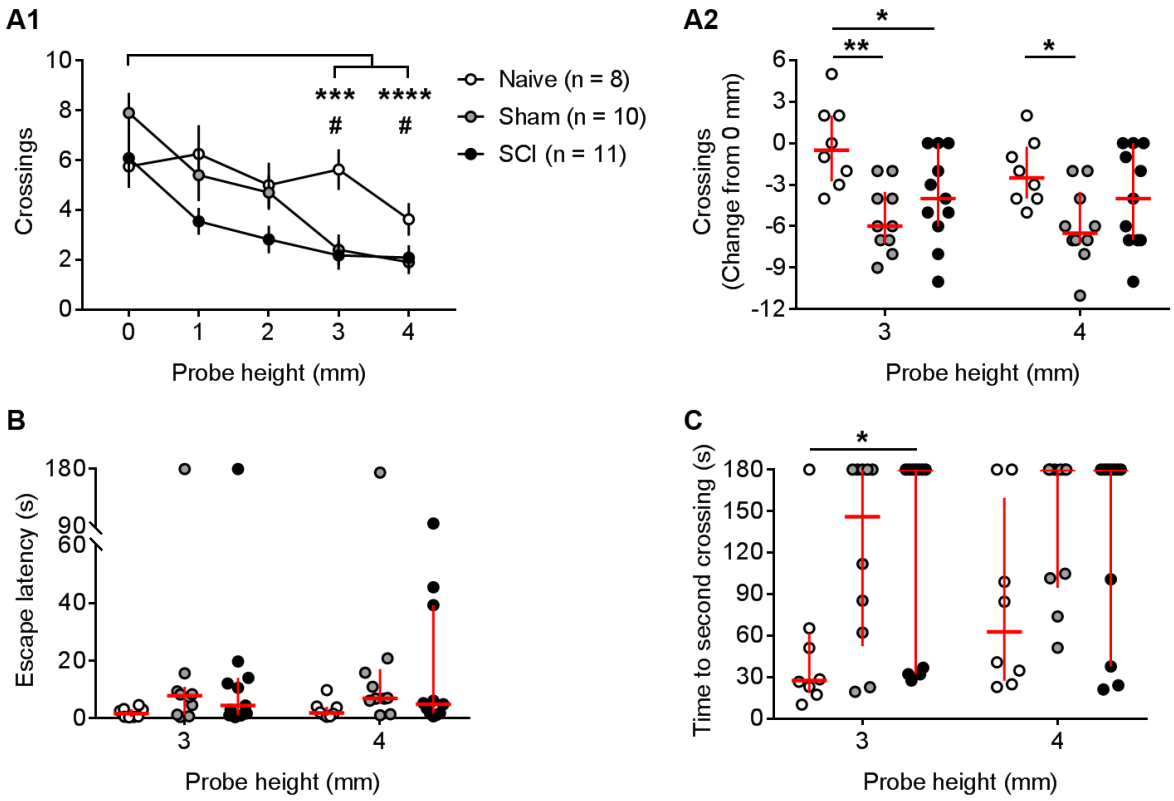


Figure 4. Spinal cord injury and its corresponding sham surgery enhance pain-avoidance behavior in a mechanical conflict test. Rats were exposed to successively ascending probe heights (0 to 4 mm). **(A1)** Crossings on 3- and 4-mm probes were decreased in sham and SCI groups. Crossings at each probe height were compared to the 0 mm baseline using a repeated measures 1-way ANOVA or Friedman test. Significance levels for sham (stars) and SCI group (pound sign) shown for Dunn's post-hoc tests. **(A2)** Planned comparisons on 3- and 4-mm probe trials revealed reductions in crossings in sham and SCI groups. **(B)** Escape latencies were not different among groups. **(C)** Some sham and SCI rats refused to cross back into the light chamber, indicated by 180-second crossing latencies. Planned comparisons in **(A2-C)** were performed using 1-way ANOVA or Kruskal-Wallis followed by Tukey's or Dunn's post-hoc test. Data shown as mean ± SEM **(A1)** and median with interquartile range **(A2-C)**. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ANOVA, analysis of variance; SCI, spinal cord injury. **Contributions:** Max A. Odem and Stephen L. Katzen designed experiments and analyzed data; Max A. Odem prepared figures; experiments were performed by Stephen L. Katzen, Kendra C. Wicks, and Emily A. Spence.

2.3.2. Standard tests for reflex sensitivity show that SCI and perhaps sham surgery can increase hindpaw heat sensitivity without increasing mechanical sensitivity

The sham surgery effect in the MC test 1-2 months post-injury raised the question of whether commonly used assays of reflex sensitivity, the von Frey mechanical sensitivity test and the Hargreaves radiant heat test, were sensitive enough to detect differences between naïve and sham rats. Hindpaw sensitivity to von Frey filaments was tested using the Chaplan/Dixon up-down method to determine 50% threshold [63,98,99], using an extended range of filaments compared to many other studies (0.4-60.0 grams, starting filament = 6 grams, modified from [90]) and log transformed for analysis and display (see [251]). No significant differences were found between the naïve, sham, and SCI groups (**Fig. 5A**). Mean non-transformed thresholds for SCI were 51 ± 5 grams, well within the range of gram forces observed in non-allodynic Sprague-Dawley rats tested using the Chaplan/Dixon up-down method (see [90]). A trend for the SCI rats' thresholds to be higher than in the naïve and sham groups (35 ± 6 grams and 35 ± 7 grams, respectively), suggests that SCI might reduce sensitivity to mechanical stimuli under these conditions, in contrast to the mechanical hypersensitivity found in previous studies (see **Table 2**). Within a randomly selected subset of rats from the samples shown in **Figure 4A**, the SCI group exhibited significantly shorter latencies for paw withdrawal to a radiant heat stimulus than the naïve group (**Fig. 5B**), while the sham group exhibited latencies intermediate between the naïve and SCI groups, but were not statistically different from either group (P = 0.06 and 0.46, respectively). These results are similar to those in previous studies, but they also suggest that sham surgery may induce a modest increase in sensitivity to noxious heat.

The absence of an SCI-induced increase in hindpaw reflex sensitivity to mechanical stimuli was unexpected because multiple groups have described SCI-induced below-level hypersensitivity in similar mid-to lower thoracic contusive injuries (see **Table 2**) (e.g., [15,16,58,66,90,130,148,151,157,196,259,396]). In principle, insufficient injury to the spinal

cord might explain the lack of mechanical hypersensitivity. Two measures were used to assess injury severity (see [58,90,196]): tissue displacement during spinal impact recorded by the impactor device (in μm) and the BBB score 1 day after injury. As expected (see [58,90,196]), our results indicated that the 50% paw withdrawal threshold decreased as contusion displacement increased, and that the rate of change (slope of the linear regression) for the threshold was in agreement with the post-SCI BBB scores (**Fig. 5C**). Contusion displacements were typical for a 150 kdyne impact [58] and internally consistent with impact data collected by the prior surgeon in the lab (data not shown). The mean displacement of 920 ± 28.8 μm and the mean BBB score of 1.0 ± 0.4 one day after SCI were also consistent with a moderate SCI [58,196], and the significant correlation between the BBB scores and contusions displacements (Spearman r = -0.503, P = 0.014) suggested these two measures were in agreement. A trending correlation between the 50% PWT and contusions displacements (Spearman r = -0.409, P = 0.052) was also observed. Moreover, all sham rats exhibited BBB scores of 21 for each hindpaw the day after surgery, indicating that unintended damage to the spinal cord during the T10 laminectomy had not occurred. Together, these data indicate that neither insufficient spinal injury in SCI rats nor inadvertent spinal injury in sham rats can explain the apparent lack of mechanical hypersensitivity in SCI rats (**Fig. 5A**). They also show that traditional reflex tests of pain may fail to reveal pain-like alterations in animals in which an operant test reveals a persistent increase in evoked pain-like behavior after either SCI or sham surgery.

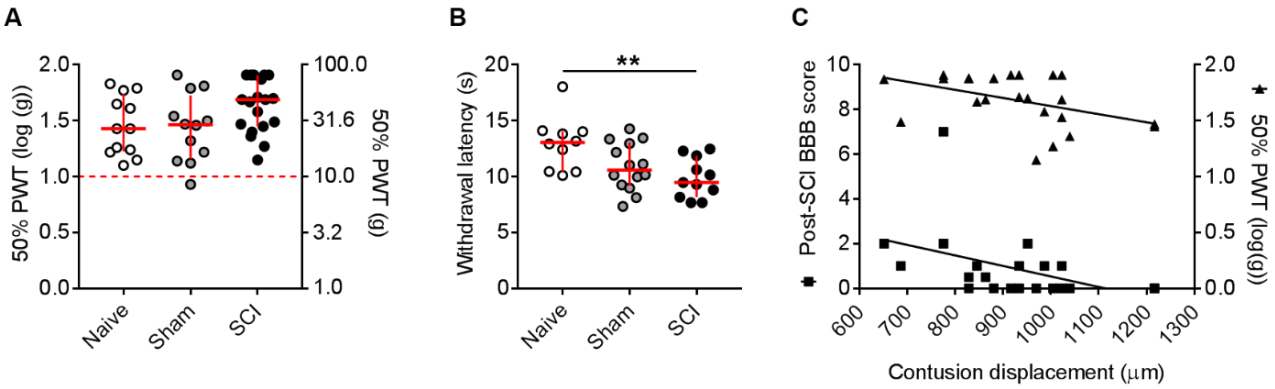


Figure 5. Standard measures of reflex sensitivity show postsurgical enhancements to heat but not weak mechanical stimuli following spinal cord injury. **(A)** The 50% PWT 1-2 months post-SCI was similar in naïve, sham, and SCI groups. Groups were compared using a 1-way ANOVA (P = 0.08). Corresponding gram forces indicated on right axis. **(B)** Withdrawal latency to a heat stimulus was lowered in SCI rats. Groups were compared using a 1-way ANOVA followed by Tukey's post-hoc test. A trend was found for lower latencies in the sham compared to naïve group (Tukey's P = 0.06). **(C)** The 50% PWT (triangles) decreased with increasing severity of the SCI, as indicated by two independent measures. Spinal cord displacement (x-axis) was measured by the controlled impactor device and the post-SCI BBB score (squares) was measured 1 day after surgery. Linear regressions: BBB scores y = -0.004706*x + 5.245, R^2 = 0.193; 50% PWT y = -0.0007266*x + 2.356, R^2 = 0.196. Data shown as median with interquartile range **(A-B)**. *P < 0.05, **P < 0.01. ANOVA, analysis of variance; BBB, Basso Beatie Bresnahan Locomotor Rating Scale; PWT, paw withdrawal threshold; SCI, spinal cord injury. **Contributions:** Max A. Odem and Stephen L. Katzen designed experiments and analyzed data; Max A. Odem prepared figures; experiments were performed by Stephen L. Katzen, Kendra C. Wicks, and Emily A. Spence.

2.3.3. Voluntary behavior during the mechanical conflict test reveals forepaw hypersensitivity in SCI rats

Rats with SCI exhibit at- and above-level mechanical hypersensitivity [28] as shown by increased forepaw sensitivity to von Frey filaments. Although one study found no correlation between SCI-induced paw hypersensitivity measured with von Frey filaments and escape latency measured in the MC test [67], our observations suggested that forepaw hypersensitivity

after injury might be expressed during voluntary behavior. Video analysis showed that the rats often pause before crossing and use their forepaws to investigate the probes. Attempts to establish weight support on the probes with their forepaws often produced a rapid withdrawal response (**Fig. 6A**). To test whether prior injury produced forepaw hypersensitivity expressed during voluntary behavior, the numbers of rapid forepaw withdrawals from the 1, 2, 3, and 4 mm probes made during initial investigation of the probes and immediately after the first crossing were counted and averaged together to increase statistical power. The SCI group showed a significant increase in the number of forepaw withdrawals compared to the naïve group (**Fig. 6B**), and the sham group was statistically indistinguishable from the other groups. In principle, each additional trial with the probes reduced their novelty and presumably the rats' drive to investigate. Thus, we also examined the number of forepaw withdrawals made during the first probe exposure (1 mm probes). The SCI group exhibited more forepaw withdraws than the naïve group, and the sham group was again statistically indistinguishable from the other groups (withdrawal number: naïve = 0.1 ± 0.1, sham = 1.8 ± 1.0, SCI = 3.5 ± 0.9; Kruskal-Wallis P = 0.02; naïve vs SCI comparison with Dunn's test P = 0.02). Trends for increased forepaw withdraws in the SCI and sham groups were found for the trials with the 2, 3, and 4 mm probes, but the trends were not statistically significant (data not shown). This indicates that operant investigations of forepaw hypersensitivity should take into consideration probe novelty. These results suggest that, when challenged with a novel, moderately noxious substrate, injured rats investigate the substrate more carefully than uninjured rats do before deciding to cross, and this investigative behavior reveals heightened sensitivity of the forepaws to noxious stimuli long after the injury.

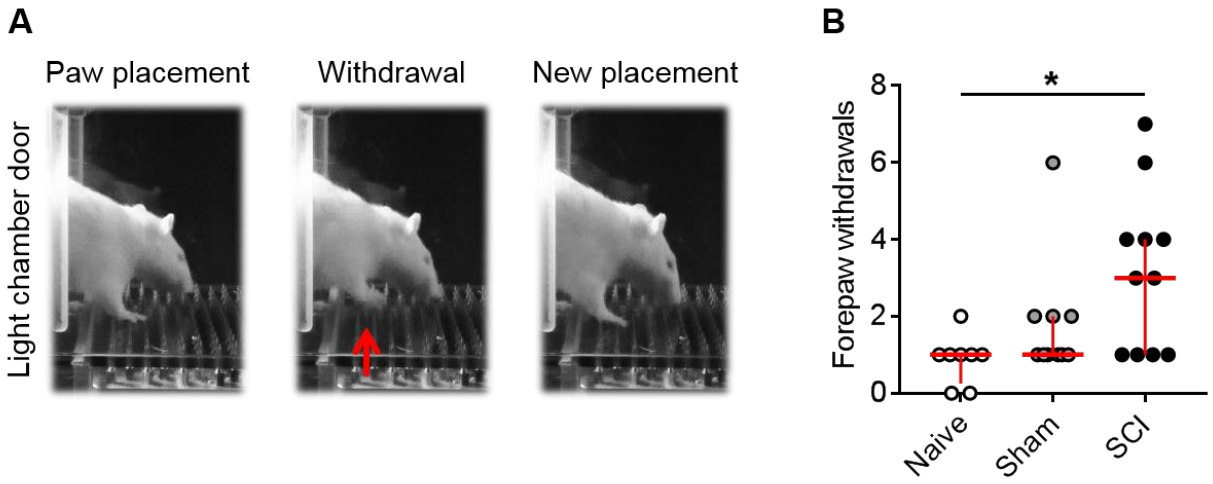


Figure 6. Rats with spinal cord injury exhibit forepaw hypersensitivity when investigating novel noxious probes before crossing the probes in the operant mechanical conflict test. **(A)** Example sequence of paw movements and probe investigation by a SCI rat prior to crossing. Rapid forepaw withdrawal – middle image, red arrow. **(B)** Rats with SCI withdrew their forepaws from the noxious probes more than naïve rats. Comparisons between groups performed using a Kruskal-Wallis test followed by Dunn's post-hoc test. Data shown as median with interquartile range. *$P < 0.05$. SCI, spinal cord injury. **Contributions:** Max A. Odem and Stephen L. Katzen designed experiments and analyzed data; Max A. Odem prepared figures; experiments were performed by Stephen L. Katzen, Kendra C. Wicks, and Emily A. Spence.

2.3.4. Pain-like probe-avoidance behavior is enhanced chronically after SCI or sham surgery

Does postsurgical enhancement of probe-avoidance behavior in SCI and sham rats persist long enough to be considered chronic? Can an increase in the novelty of noxious probes differentiate probe-avoidance behavior in SCI and sham-operated rats? To address these questions, naïve, sham, and SCI rats were examined 3 to 6 months after injury using a shortened testing protocol in which rats only encountered the probes (4 mm) twice (see **Figure 2**, second SCI timeline). The day before exposure to the probes all rats showed similar exploratory behavior and crossings of the middle chamber during 3 familiarization trials, and

there was no significant difference among the groups on the 0 mm baseline trial on the day of probe exposure (**Figs. 7A1**). All groups showed decreases in the number of crossings on the probes compared to their baseline crossings without the probes, and this effect was significant for each group during the second 4 mm probe exposure. Planned comparisons showed that both the SCI and sham rats crossed the 4 mm probes fewer times than the naïve rats during the first exposure to the probes, and during the second exposure the SCI rats showed significantly less crossing than the naïve rats (**Figs. 7A2**). Scatter plots of the number of crossings (**Figs. 7A2**) and escape latencies (**Fig. 7B**) show that half the rats in the sham group and all the rats in the SCI group refused to cross the probes even once during the second probe exposure, whereas all the naïve rats crossed and 5 of 8 of the naïve rats crossed multiple times. Forepaw withdrawals elicited by 4 mm probes were compared in rats during their first exposure to probes. Both the sham and SCI groups exhibited significantly more forepaw withdrawals than the naïve group (**Fig. 7C**). Comparisons of forepaw withdrawals during the second exposure to the 4 mm probes were not performed because many of the rats in the SCI group completely avoided the probes, staying on the opposite side of the light chamber and facing away from the probes. These observations indicate both the SCI and sham surgery increase the aversiveness of the probes and enhance pain-avoidance behavior chronically (≥3 months post-surgery).

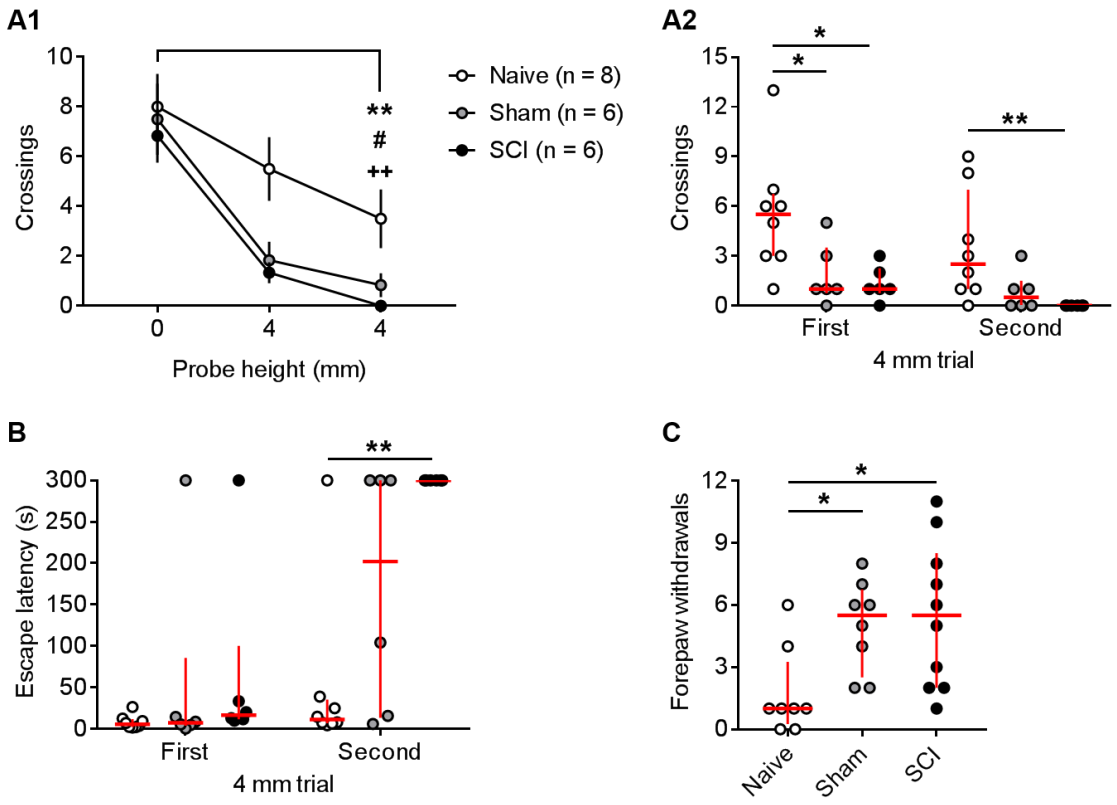


Figure 7. Spinal cord injury and its corresponding sham surgery chronically enhance pain-avoidance behavior in the mechanical conflict test. **(A1)** Significantly reduced crossings in naïve, sham, and SCI groups during the second 4-mm probe trial. **(A2)** Planned comparisons between groups showed sham and SCI groups made fewer crossings than naïve rats on the first probe trial, and SCI rats crossed crossings fewer times than naïve rats on the second probe trial. All SCI rats (6 out of 6) and 50% of shams (3 out of 6) refused to cross at the second probe trial, while only 13% of naïve rats (1 out of 8) refused. **(B)** Escape latencies on the second probe trial reflected crossing results in **(A2)**. **(C)** Forepaw withdrawals were increased in sham and SCI groups during the first 4-mm probe trial. Crossings for groups during both probe trials in **(A1)** were compared to the 0 mm baseline using the Friedman test. Significance levels for naïve (stars), sham (pound sign), and SCI group (plus sign) shown for Dunn's post-hoc tests. Planned comparisons between groups **(A2-C)** were performed using a 1-way ANOVA or Kruskal-Wallis test followed by Tukey's or Dunn's post-hoc tests. Data shown as mean ± SEM **(A1)** and median with interquartile range **(A2-C)**. *P < 0.05, **P < 0.01. ANOVA, analysis of variance; SCI, spinal cord injury. **Contributions:** Max A. Odem and Stephen L. Katzen designed experiments and analyzed data; Max A. Odem prepared figures; experiments were performed by Stephen L. Katzen, Tamara McGhee, and Emily A. Spence.

2.3.5. Probe-avoidance behavior is enhanced by sham surgeries for peripheral nerve injury models

The studies above show that surgical damage to tissues, including muscle and bone, required to expose the spinal cord for controlled contusive injury was sufficient to persistently enhance avoidance of noxious probes. This hyperalgesic effect was shown more clearly by the MC test than by the von Frey reflex test, suggesting that the MC test may be a more sensitive test for pain evoked by mechanical stimuli. We asked whether the MC test might also reveal hyperalgesia produced by the sham surgeries used for common PNI models that also require damage to tissues (e.g., muscle retraction, nerve manipulation) that can contribute to inflammation and pain [40,122,131,197]. While the MC test has shown that rats with a CCI of the sciatic nerve exhibit prolonged escape latencies [155], this study did not compare MC tests and von Frey tests, or include sham controls. Mice with SNI-induced allodynia (assessed with von Frey tests) also exhibit longer escape latencies [326], but this study did not compare the SNI mice to appropriate sham controls in the MC test. Thus, whether peripheral sham surgery is sufficient to enhance pain-avoidance behavior in the MC test and whether the von Frey reflex test is a good predictor of pain-avoidance behavior after hindlimb surgery are unknown.

We used two PNI models to address these questions for either acute (days) or subacute (weeks) pain. One is the L5 SNT [338,339,345] along with its sham surgery procedure, which is identical to the sham surgery used as a control for SNL models [163]. The second is the sciatic nerve CCI model along with its sham surgery procedure [31,137,138].

In the SNT experiments, rats were tested for exploratory behavior (crossings) <1 week before and 1 day post-surgery, and then exposed to the 4 mm probes after the final 0 mm trial 3 days post-surgery (see **Figure 2**, SNT timeline). Interestingly, the SNT group exhibited a significant reduction in crossings compared to its pre-injury number when tested without probes 3 days after injury (**Fig. 8A1, A2**). In contrast, the naïve and sham rats showed little or no

change in crossings in the absence of probes. The effect at 0 mm in the SNT rats might represent modest motor impairment or extreme allodynia that discourages locomotion on the allodynic limb. All groups showed a reduction in crossings on the 4 mm probes, with the largest difference from the pretests in the SNT group, while the sham group was not significantly different from the SNT or naïve groups. As in the SCI experiments, the escape latencies (**Fig. 8B**) did not distinguish the three groups. In addition, the SNT group developed a robust mechanical hypersensitivity in the hindpaw ipsilateral to the injury (**Fig. 8C** left panel, ~4 g threshold in the SNT group versus ~12 g in the naïve and sham groups, before log transforming), but not in the contralateral hindpaw (**Fig. 8C** right panel).

In the CCI experiments, we tested rats 14 days post-surgery, a time when reported mechanical allodynia is well established [137,138]. All groups showed significantly reduced crossings during both 4 mm probe trials compared to their baseline crossings (**Fig. 9A1**). While no significant differences among the groups were observed for the first 4 mm trial, the sham and CCI groups crossed significantly fewer times than the naïve during the second 4 mm trial (**Fig. 9A2**). There were trends for longer escape latencies in the sham and CCI groups (**Fig. 9B**), with some rats crossing back and forth over the probes freely while others refused to cross even once. In contrast to the robust effects found in the sham group in the MC test, no evidence of mechanical hypersensitivity in the sham group was found with the von Frey test (**Fig. 9C**). As expected, CCI produced strong mechanical hypersensitivity in the hindpaw ipsilateral to the injury (**Fig. 9C**, left panel).

In sum, sham-operated rats for both types of PNI models failed to exhibit mechanical hypersensitivity in von Frey tests that provided evidence for allodynia in SNT and CCI rats, yet both sham groups showed clear evidence of enhanced pain-avoidance behavior in the MC test.

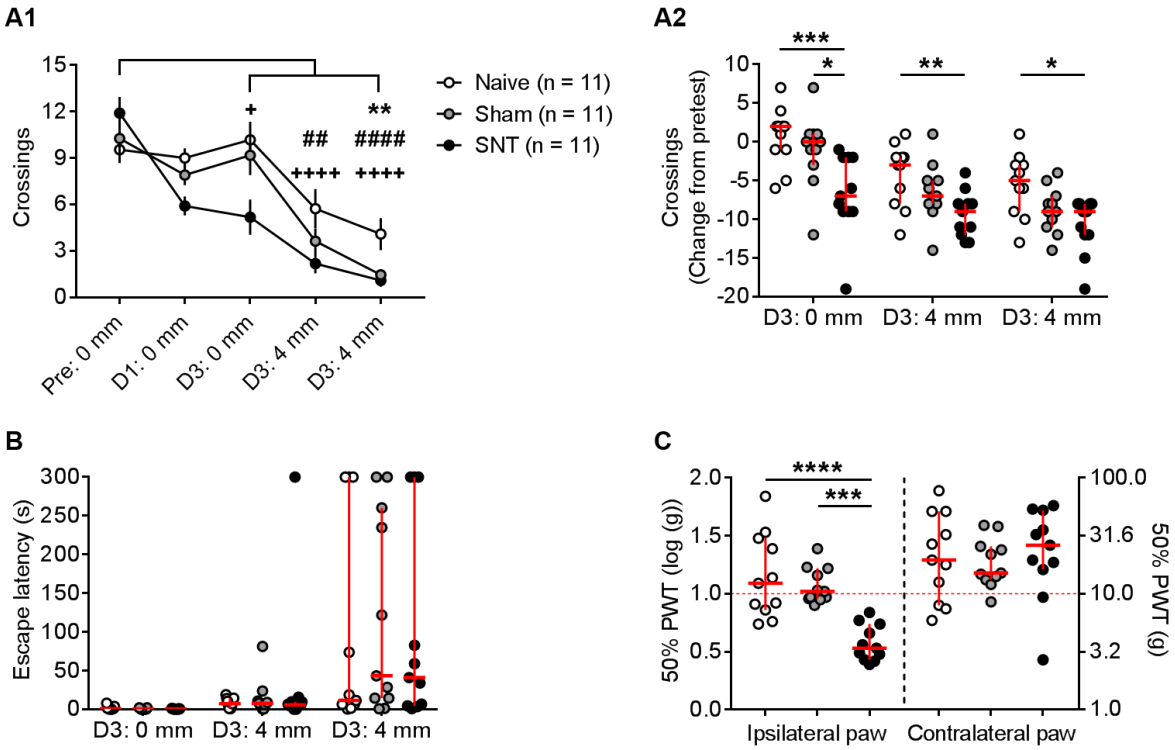


Figure 8. Spinal nerve transection and sham surgery enhance pain-avoidance behavior in the mechanical conflict test 3 days after injury. **(A1)** All groups exhibited a reduction in crossings on the 4-mm probes. Crossings at each post-surgery trial were compared to the pre-surgery 0-mm baseline using the Friedman test. Significance levels for naïve (stars), sham (pound sign), and SNT group (plus sign) shown for Dunn's post-hoc tests. **(A2)** Rats with SNT showed reduced crossings in the absence of the probes during the 0-mm baseline trial on day 3. The SNT rats had fewer crossings during both probe trials compared to naïve rats. The sham group was statistically indistinguishable from SNT and naïve groups. **(B)** Escape latencies did not reveal differences between groups. **(C,** left panel**)** The 50% PWT of the hindpaw ipsilateral to the side of injury was reduced in rats with SNT, but not on the contralateral (uninjured) hindpaw, while PWT measures for either hindpaw did not differ between sham and naïve groups **(C,** right panel**)**. Corresponding gram forces shown on right axis. Planned comparisons between groups in **(A2-C)** were performed using a 1-way ANOVA or Kruskal-Wallis test followed by Tukey's or Dunn's post-hoc tests. Data shown as mean ± SEM **(A1)** and median with interquartile range **(A2-C)**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. ANOVA, analysis of variance; PWT, paw withdrawal threshold; SCI, spinal cord injury; SNT, spinal nerve transection. **Contributions:** Max A. Odem and Stephen L. Katzen designed experiments and analyzed data; Max A. Odem prepared figures; experiments were performed by Stephen L. Katzen, Kendra C. Wicks, and Emily A. Spence.

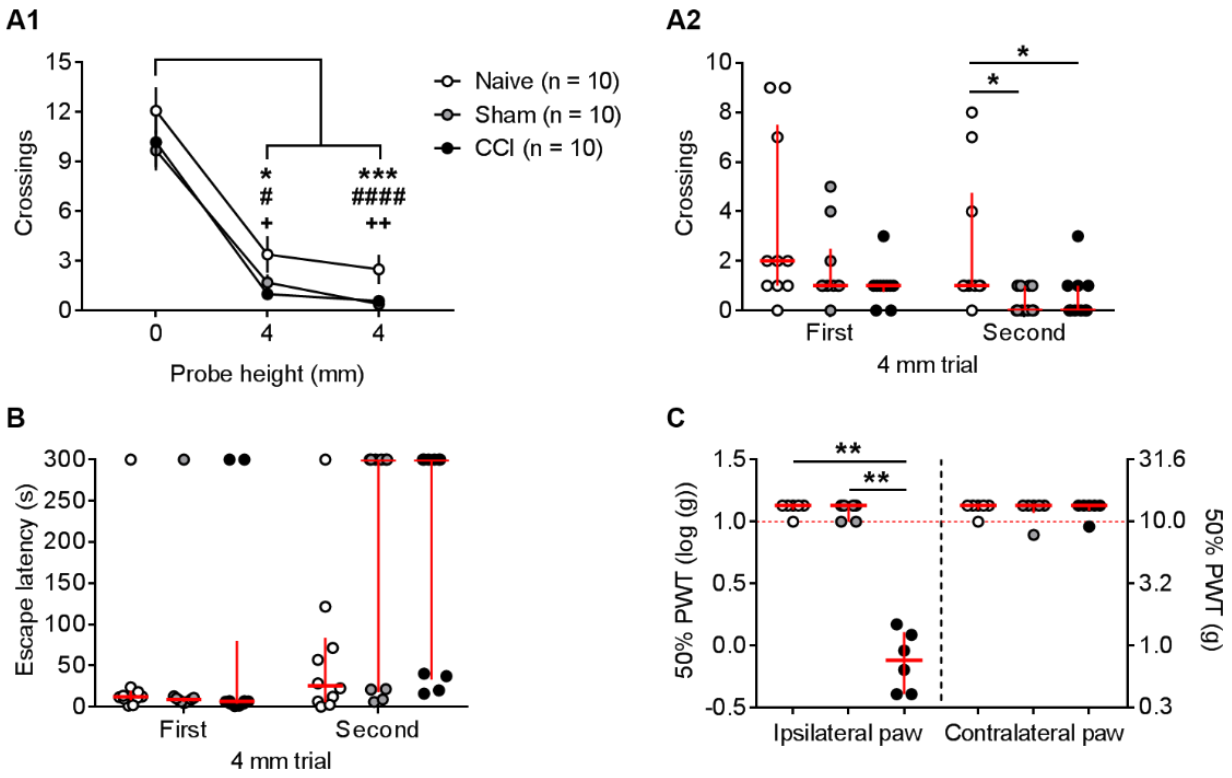


Figure 9. Chronic constriction injury of the sciatic nerve and its corresponding sham surgery enhance pain-avoidance behavior in the mechanical conflict test. **(A1)** All groups exhibited a reduction in crossings during both 4-mm probe trials compared to the 0-mm baseline (Friedman test). Significance levels for naïve (stars), sham (pound sign), and CCI group (plus sign) shown for Dunn's post-hoc tests. **(A2)** No significant differences in crossings were observed on the first probe trial, but sham and CCI groups crossed fewer times than naïve rats during the second probe trial. **(B)** Escape latencies did not reveal differences between groups. **(C,** left panel**)** The 50% PWT of the hindpaw ipsilateral to the side of injury was reduced in rats with CCI, but not on the contralateral (uninjured) hindpaw **(C,** right panel**)**. Note that 50% PWT measures <1 gram are negative after log transformation. Corresponding gram forces shown on right axis. Planned comparisons between groups in **(A2-C)** were performed using a 1-way ANOVA or Kruskal-Wallis test followed by Tukey's or Dunn's post-hoc tests. Data shown as mean ± SEM **(A1)** and median with interquartile range **(A2-C)**. **P* < 0.05, ***P* < 0.01. ANOVA, analysis of variance; CCI, chronic constriction injury; PWT, paw withdrawal threshold; SCI, spinal cord injury; SNT, spinal nerve transection. **Contributions:** Michael J. Lacagnina, Jiahe Li, Peter M. Grace, and Max A. Odem designed experiments; Michael J. Lacagnina and Max A. Odem analyzed data and prepared figures; experiments were performed by Michael J. Lacagnina and Jiahe Li.

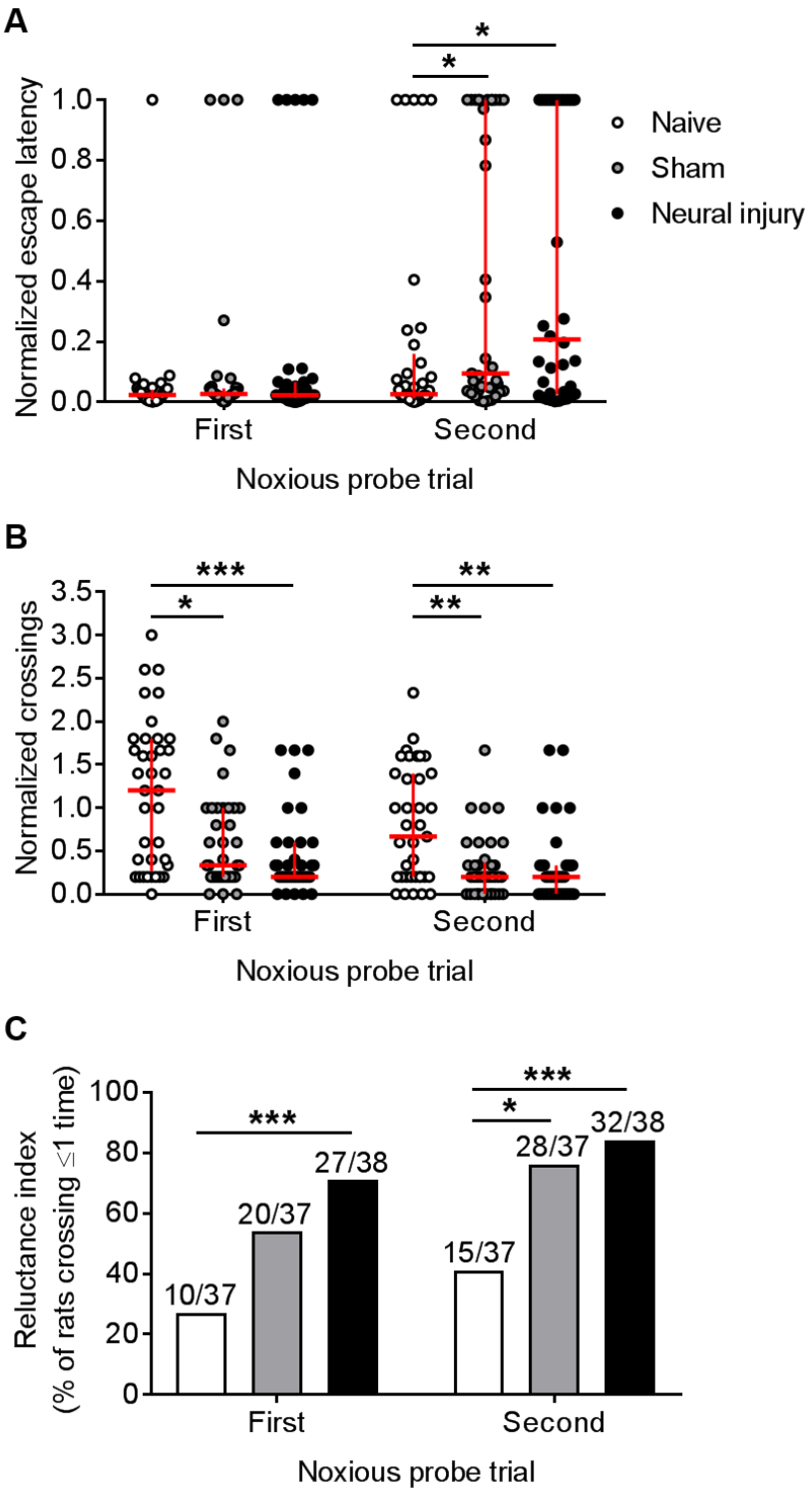
2.3.6. Increased reluctance to repeatedly cross noxious probes provides a more sensitive indicator of enhanced evoked pain than latency to escape the light chamber

In contrast to the present study, previous studies utilizing the MCS greatly reduced the rodents' exploratory drive by giving many familiarization and training trials before introducing the probes during test trials. Moreover, these studies removed the rodent after a single crossing of the probes (not permitting multiple crossings), and usually used the latency to escape from the light chamber as their measure of pain avoidance [67,143,155,210,271,326]. Our results with each of the three neuropathic pain models and their corresponding sham surgeries suggested that, in rodents that have not been extensively familiarized with the MCS, the number of crossings of the noxious probes (presumably motivated by a drive to explore the MCS, see [113]) is a more sensitive measure of the aversiveness of the probes than is the initial escape latency. To test this possibility with greater statistical power, we took advantage of the fact that each of our studies had the same basic design, including limited familiarization trials, identical measures of escape latency and multiple crossings, and the inclusion of naïve, sham-operated, and neural injury groups. Thus, we combined corresponding groups from each study, with the escape latencies and crossings from SCI, SNT, and CCI rats and their sham controls pooled into separate neural injury and sham groups, which were compared to the pooled naïve group.

Normalized escape latencies (**Fig. 10A**) only revealed significant increases in the neural injury and sham groups during the second exposure to noxious probes (4 mm in all studies, plus 3 mm from the study in **Figure 4**). Escape latencies (normalized to test duration) in the neural injury and sham groups showed a clear bimodal distribution, especially during the second noxious probe trial, which cannot be captured by the measure of central tendency (mean or median) that is usually reported. In contrast to the escape latencies, the number of crossings (normalized to test duration) on both the first and second noxious probe trials were significantly decreased in the neural injury and sham groups (**Fig. 10B**). Additional information

about the effects of neural and postsurgical injury on the aversiveness of the probes was shown by the relative reluctance of rats in each group to cross the probes more than once. The neural injury group and (on the second noxious probe trial) the sham group were significantly more reluctant to cross the probes two or more times than naïve rats (**Fig. 10C**). These results confirm that commonly used sham surgeries in rats induce persistent hypersensitivity to noxious probes that appears to enhance pain-avoidance behavior, and they show that previously unrecognized pain-related effects can be revealed by a reduction of exploratory behavior on a noxious substrate.

Figure 10. Reluctance to make multiple crossings over noxious probes in the mechanical conflict test is a sensitive measure of pain-avoidance behavior. Escape latencies and crossings measured during noxious probe trials (first 3- or 4-mm trial and second 4- mm trial) from all naïve, sham-operated, and rats with neural injury (SCI, SNT, and CCI) were pooled into 3 separate groups. Escape latencies and crossings were normalized to test duration. **(A)** Normalized escape latencies were not different on the first probe trial, but latencies were greater in sham and neural injury groups on the second trial. Bimodal distributions reflect the refusal of many rats to cross during the second trial. **(B)** Normalized data reveal reduced crossings during both trials by sham and neural injury groups compared to naïve. **(C)** Indexing rats by their reluctance to make multiple crossings reveals a significant increase in the percentage of rats with neural injury that refused to cross on the first trial, and on the second trial the percentage of rats in both sham and neural injury groups that refused to cross was greater than in the naïve group. Planned comparisons between groups in **(A-B)** were performed for using a 1-way ANOVA or Kruskal-Wallis test followed by Dunnett's or Dunn's post-hoc tests, and planned comparisons between proportions in **(C)** were performed using Fisher exact tests with Bonferroni corrections for multiple comparisons. Data shown as median with interquartile range **(A-B)** and fractions above bars represent number of rats/total number in group **(C)**. *P < 0.05, **P < 0.01, ***P < 0.001. ANOVA, analysis of variance; CCI, chronic constriction injury; SCI, spinal cord injury; SNT, spinal nerve transection. **Contributions:** Max A. Odem, Michael J. Lacagnina, Stephen L. Katzen, Jiahe Li, and Peter M. Grace designed experiments; Max A. Odem, Michael J. Lacagnina, and Stephen L. Katzen analyzed data; Max A. Odem prepared figures; experiments were performed by Michael J. Lacagnina, Stephen L. Katzen, Jiahe Li, Kendra C. Wicks, and Emily A. Spence.


A
*
*
Normalized escape latency
1.0
0.8
0.6
0.4
0.2
0.0
Naive
Sham
Neural injury
First
Second
Noxious probe trial
B
***
*
**
**
Normalized crossings
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
First
Second
Noxious probe trial
C
***
***
*
Reluctance index
(% of rats crossing ≤1 time)
100
80
60
40
20
0
10/37
20/37
27/38
15/37
28/37
32/38
First
Second
Noxious probe trial

2.4. Conclusions and significance

Surprisingly, the most significant, novel finding in this study was that sham surgical procedures for three neuropathic pain models (SCI, SNT, and CCI) were sufficient to enhance evoked pain-avoidance behavior for periods lasting days, weeks, and months after surgery. This is the first known study to compare crossings across noxious probes in the MC test as a sensitive measure for postsurgical and neuropathic pain-related alterations in rats' innate drive to explore novel environments. Furthermore, reflex sensitivity to innocuous mechanical stimulation was not a strong predictor of sham-induced hyperalgesia in the MC test; von Frey tests revealed mechanical hypersensitivity in SNT and CCI rats, but all sham and neural injury groups exhibited pain-avoidance behavior. While von Frey tests failed to reveal below-level mechanical hypersensitivity following SCI, rats exhibited below-level heat hypersensitivity as well as forepaw hypersensitivity that was revealed using a novel measure of voluntary investigation of noxious probes in the MC test.

These findings have major implications for future studies that address mechanisms associated with neuropathic pain as one particular clinically relevant dimension (i.e., postsurgical pain) is underappreciated and not always explicitly investigated [75,186]. Many pain-related studies use similar rodent injury models and reflex tests to recapitulate neuropathic conditions. Both clinical and animal data show that postsurgical pain is greater for deeper, more extensive incisions [64,137,237]. Sham surgeries for most nerve injury models, including the SNT, SNL, and CCI models, produce deep tissue damage (see also [122]). Postsurgical pain is a pervasive problem that covers a breadth of different surgeries and conditions [75,172,297,347,363], and it is not uncommon for human patients to develop severe pain following surgery [114,127]. The underlying mechanisms [64,186] and time course for postsurgical pain can mirror that of chronic pain that persists ≥3 months [347,368].

Chapter 3: *Ongoing pain is important after neural injury*

The novel observation of sham-induced hyperalgesia in the MC test suggests behavioral tests for evoked pain may not be appropriate for distinguishing pain-related alterations due to neural injury from postsurgical effects. Persons with chronic neuropathic and inflammatory conditions often describe ongoing pain as being debilitating compared to evoked pain [30]. Behavioral tests that capture the negative qualities of ongoing pain might be better suited for reflecting this clinical reality. Ongoing pain can be difficult to assess in rodents in contrast to evoked pain (for review see [344]), but progress using operant methods like the CPP test [256,286] has revealed ongoing pain in rodents with SCI [389], PNI [19,142,159,192,291,380], paw incision [85,257,380], inflammation and arthritis [159,227,265,277], cancer-induced bone pain [158], diabetic neuropathy [353], and chemotherapy-induced peripheral neuropathy [179,278]. These studies show naïve, sham, and other pertinent controls do not exhibit a strong preference for analgesic-paired chambers, suggesting the absence of ongoing pain. These studies demonstrate neuropathic and inflammatory conditions selectively produce ongoing pain.

First-line treatments for patients with neuropathic pain often include gabapentinoids, like gabapentin or pregabalin (brand names Neurontin™ and Lyrica™, respectively). Gabapentin is commonly used in preclinical pain studies to promote CPP [19,142,179,278]. It is not innately rewarding [12] and appears to be exceptionally powerful, it produces CPP in rats with SNL after single-trial conditioning (i.e., one exposure to the unconditioned stimulus) [19]. I have wanted to optimize CPP procedures to facilitate drug testing on SCI-induced ongoing pain ever since my contribution to the CPP experiments described in [389] (CPP with the anticonvulsant retigabine was produced by 3 conditioning trials). The Bannister *et al*. study [19] suggests gabapentin might have robust effects on ongoing neuropathic pain which would assist in optimizing CPP for future studies using SCI. However, no studies have described gabapentin-produced CPP in SCI models despite the effectiveness of gabapentinoids to reduce SCI-induced above-level

hypersensitivity [171], spasticity [194], anxiety [14], and to lessen escape/avoidance behavior in the PEAP test [15]. In light of this, I predicted gabapentin might also be effective at producing CPP in SCI rats, but not naïve or sham controls. Surprisingly, single-trial conditioning with gabapentin (GBP, 100 mg/kg, i.p., saline vehicle; see *Appendix* for methods) was sufficient for rats with SCI to develop a significant preference for the GBP-paired chamber (see **Fig. 11**). Note that naïve and sham rats do not prefer the GBP-paired chamber. This is quite promising as a shortened CPP protocol can be used as a backdrop for relatively efficient screening of other experimental drugs that might reduce SCI-induced ongoing pain (see [25,389]).

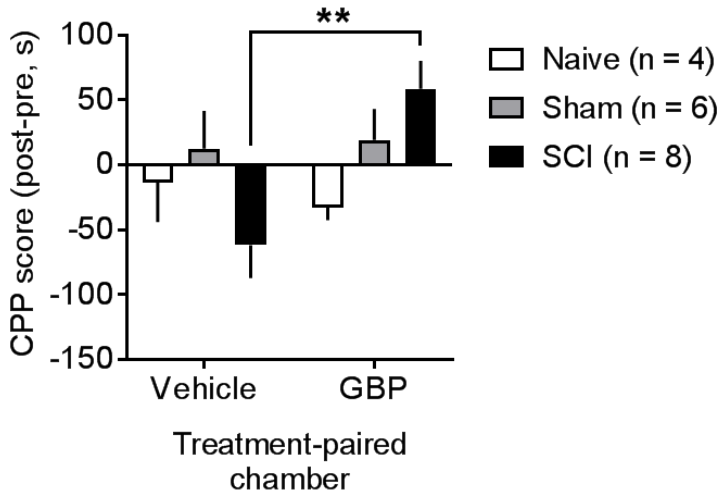


Figure 11. Single-trial conditioning with gabapentin selectively promotes conditioned place preference in rats with spinal cord injury, not in naïve and sham-operated rats. ***P* < 0.01. CPP, conditioned place preference; GBP, gabapentin; SCI, spinal cord injury. **Contributions:** Max A. Odem designed experiments, analyzed data and prepared the figure; Stephen L. Katzen and Emily A. Spence performed experiments.

The precise mechanism of action for gabapentin is not fully understood, but it is thought to reduce neuronal transmission at presynaptic terminals in the dorsal horn and potentially attenuate contribution of nociceptor OA to spinal sensitization and maintenance of ongoing pain in neuropathic conditions (for review [203]). Indeed, ongoing pain-related behaviors and central sensitization are dynamically driven by ectopic/OA generated in primary afferents [4,92,139,153,209,225,244,260,285]. Multiple signals for injury and inflammation integrate at the level of the DRG to promote a hyperfunctional state in nociceptors [356,358]. The Walters Lab group has demonstrated a majority of small-diameter dissociated DRG sensory neurons (putative nociceptors) enter into a hyperfunctional state and generate SA *in vitro* following contusive SCI [25,28,377,389]. Importantly, SCI-induced gross and single-unit C-fiber SA is generated in/near DRGs *in vivo* [28], further suggesting nociceptor somata are critical sources of SA that might drive central sensitization and ongoing pain. The tetrodotoxin-resistant (TTX) Na^+ voltage-gated channel Nav1.8 is preferentially expressed in unmyelinated DRG sensory neurons [349] – many of which are nociceptors – and is important for AP generation [207,302,378]. Knockout or pharmacological blockade of Nav1.8 reduces SA generated in excised neuromas [306] and small-diameter dissociated DRG sensory neurons [174], respectively. Expression of Nav1.8 protein increases in DRGs following SCI, and selective knockdown of Nav1.8 (presumably in DRGs) *in vivo* using antisense oligodeoxynucleotides blocks CPP in SCI rats as well as nociceptor SA *in vitro* [389]. These studies strongly suggest nociceptor activity is critical for maintenance of ongoing neuropathic pain. Human/rodent microneurography studies [195,262,263,269,270,322–324] also link ongoing pain to OA generation in C-fiber nociceptors rather than OA generated in A-fibers. While the processes underlying OA in A-type neurons are well established (e.g., sinusoidal oscillations in membrane potential, see [7–9,223,224,384]), the neurophysiological basis for OA in C-type nociceptors remains largely unknown. Furthermore, *in vitro* investigations of firing properties in nociceptors using whole-cell current clamp recordings often utilize large, rapid, relatively brief current injections to depolarize neurons that do not permit reliable assessments of any sustained OA.

Wu *et al*. demonstrated that extremely low concentrations of capsaicin (~10 nM) are sufficient to promote sustained OA and speculate on possible mechanisms (e.g., oscillations in membrane potential) [377], but there has not been a thorough, quantitative analysis of any regular and/or irregular activity in membrane potential in nociceptors (see [239,337]). The rat thoracic contusive SCI model offers a unique opportunity to investigate the neurophysiological basis of nociceptor OA under conditions when ongoing pain is known to be present and a majority of nociceptors generate true SA [25,28,377,389]. In the next chapter I will address the following questions to describe the neurophysiological basis of nociceptor OA:

Study 1: Are all putative nociceptors specialized to generate OA *in vitro*?

Study 2: What are the electrophysiological signatures that define nociceptor OA?

Study 3: Can nociceptor OA be potentiated under conditions other than SCI?

Chapter 4: *Isolated nociceptors reveal multiple specializations for generating irregular ongoing activity associated with ongoing pain*

Disclosure: This chapter is based upon: Max A. Odem, Alexis G. Bavencoffe, Ryan M. Cassidy, Elia R. Lopez, Jinbin Tian, Carmen W. Dessauer, Edgar T. Walters, Isolated nociceptors reveal multiple specializations for generating irregular ongoing activity associated with ongoing pain, *Pain* 159 (11):2347-2362. Portions of the text, results, and figures are granted gratis to the first author with no formal licensing from Wolters Kluwer Health, Inc., July 26, 2018. Copyright © 2018, © 2018 International Association for the Study of Pain.

Study 1: *One class of dorsal root ganglion sensory neurons is specialized for generating ongoing activity*

4.1. Rationale

Whole-cell recordings from isolated DRG neurons offer powerful insight into the neurophysiology and function of individual neurons. For example, application of algogenic substances (e.g., capsaicin, serotonin) activates nociceptors and evoke bursts of sustained firing of APs and/or can sensitize nociceptors to other types of inflammatory agents [132,133,161,296]. The electrophysiological properties observed *in vitro* are likely to represent similar functions maintained *in vivo* in the soma and/or in the peripheral terminals [17,154,307]; indeed, injection of inflammatory mediators in the paw of rodents evokes hyperalgesic responses and spontaneous pain-like behaviors [166,301,341]. Notable electrophysiological properties that have been used to distinguish various subpopulations of DRG neurons include membrane properties such as the capacitance or transmembrane potential, chemical-evoked currents, voltage-dependent currents, and AP kinetics [70,102,104,106,132,156,282,283,302,315,398], all of which are often described within subpopulations of neurons delineated by soma diameter. But classification of DRG sensory neurons *in vitro* depends upon a myriad of additional anatomical, molecular, and

electrophysiological properties [23,117,211,268,282,283,336] across multiple species and phyla [331]. Genetic-editing tools reveal distinct subpopulations of nociceptors based upon anatomical projections, sensory modalities, and behavioral function *in vivo* (for example see [61] and for review see [284]). There are multiple subpopulations of sensory neurons with unique and overlapping gene expression profiles that may provide clues about sensory function [349] in different pain conditions [276,299]. These studies, and certainly many others, have greatly expanded our appreciation for sensory neuron heterogeneity and provide useful roadmaps for mechanistic analyses. It is generally accepted that the small diameter ≤30 μm DRG sensory neurons primarily represent overlapping subpopulations of unmyelinated C-type nociceptors while medium and large diameter >30 μm DRG sensory neurons are comprised of myelinated A-type neurons that are important for touch and proprioception, but also include some nociceptors.

The major goal of these experiments was to characterize subpopulations of dissociated DRG sensory neurons *in vitro* based on functional capacity for sustained OA. *In vitro* whole-cell recordings of small-diameter (15-30 μm) DRG neurons dissociated from naïve rats were used to provide direct access to the neuron soma, which retains properties observed *in vivo* [17,132,154]. Series of prolonged depolarizing pulses (2 s sweeps, Δ5 pA increments) under current clamp were used to trigger possibly sustained OA during steady-state inactivation of most voltage-gated Na^+ channels [70,156] and to assess any potential electrophysiological properties that would suggest neurons are specialized for generating sustained OA. Preliminary experiments indicated that one subpopulation of small dissociated DRG neurons was capable of sustained OA while another was not. The following experiments define the electrophysiological properties of those two subpopulations of probable nociceptors.

4.2. Materials and methods

4.2.1. Animals

All procedures followed the guidelines of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee for the University of Texas Health Science Center at Houston (UTHealth). Male, Sprague-Dawley rats (Envigo, USA) were housed at McGovern Medical School, the rats (250-300 g, 2 per cage) were acclimated to a controlled environment (12-hour reverse light/dark cycle, 21 ± 1°C) for ≥4 days before beginning experiments. The corn cob bedding was replaced 2-3 times per week while food and water were provided *ad libitum*.

4.2.2. Dissociation and culture of dorsal root ganglion neurons

Rats were euthanized using pentobarbital/phenytoin (0.9 ml; Euthasol, Virbac AH, Inc., Fort Worth, TX) followed by transcardial perfusion of ice-cold phosphate buffered saline (Sigma-Aldrich, St. Louis, MO). DRGs were excised from spinal segments T11 to L6 and incubated at 34°C for 40 minutes with trypsin (0.3 mg/ml) and collagenase D (1.5 mg/ml) enzymes in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich). Following digestion and washing the DRG fragments were mechanically triturated in DMEM with a fire-polished Pasteur pipette and plated on 8 mm glass coverslips coated with poly-L-ornithine (Sigma-Aldrich). Dissociated neurons were incubated overnight (<5% CO_2, 95% humidity, 37°C) in DMEM without serum, growth factors, or other supplements.

4.2.3. Whole-cell recordings from dissociated dorsal root ganglion neurons

Small DRG neurons (soma diameter ≤ 30 μm) were recorded on glass coverslips at room temperature, 18-30 hours after dissociation, on either a Zeiss Axiovert 200M or Olympus IX71 inverted microscope with 40X or 20X magnification, respectively. The bath was filled with extracellular solution containing (in mM): 140 NaCl, 3 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and

10 glucose, which was adjusted to pH 7.4 with NaOH and 320 mOsM with sucrose. HEKA EPC10 amplifiers (HEKA Elektronik, Lambrecht/Pfalz, Germany) were used for whole cell patch clamp recordings. Data were sampled at 20 kHz with PatchMaster v2x90.1 (HEKA Elektronik) and filtered with a 10 kHz Bessel filter. Borosilicate glass capillaries with outer diameter of 1.5 mm and inner diameter of 0.86 mm (Sutter Instrument Co., Novato, CA) were pulled using a Sutter P-97 Flaming/Brown Micropipette Puller. Fire-polished patch pipettes had electrode resistances of 3-8 MΩ after filling with intracellular-like solution containing (in mM): 134 KCl, 1.6 $MgCl_2$, 13.2 NaCl, 3 EGTA, 9 HEPES, 1 Mg-ATP, and 0.3 Na-GTP, which was adjusted to pH 7.2 with KOH and 300 mOsM with sucrose. Only neurons that were not in visible contact (at 20-60x magnification without any staining) with the somata or neurites of other neurons, or debris, were selected for whole-cell recording. Membrane resistance and capacitance were measured under voltage clamp using 5 ms, 5 mV depolarizing pulses from a holding potential of -60 mV. To permit direct comparison with previous papers, the liquid junction potential (calculated to be ~4.3 mV) was not corrected. This means that actual membrane potentials were probably ~4 mV more negative than all values reported. To measure SA, neurons were recorded under current clamp at resting membrane potential (RMP; 0 current injected) for at least 1 minute beginning at least 1 minute after switching from voltage clamp. Next, membrane potential was set at -60 mV with a constant holding current under current clamp while a series of depolarizing current injections (2 second steps every 4 seconds, +5 pA increments) were used to measure rheobase, latency to the first AP at rheobase, the membrane time constant (τ), the AP voltage threshold, and any repetitive firing at rheobase or 2x rheobase. In some experiments neurons were held at -45 mV under current clamp for ≥30 second to facilitate OA. A subset of neurons was held at -60 mV and single APs were evoked by 2 ms depolarizing pulses (+20 pA increments) to measure AP and afterhyperpolarization (AHP) properties (modified from [398]).

4.2.4. Markers for nociceptive function

A majority of nociceptors express the transient receptor potential vanilloid-1 (TRPV1) non-selective cation channel and/or bind the non-peptidergic marker isolectin B4 (IB4) [60,132,268,282–284,336,377]. At the end of some experiments neurons were superfused with 1 µM capsaicin (dissolved in extracellular solution; Sigma-Aldrich, St. Louis, MO, USA) using a gravity-fed delivery system made with polyimide tubing (0.36 mm outer diameter, 0.31 mm inner diameter; Cole-Parmer, Vernon Hills, IL, USA) positioned ~600 um away from each neuron. Sensitivity to capsaicin was assessed under voltage or current clamp and a positive response was confirmed by the presence of capsaicin-evoked inward currents or depolarization and excitation, respectively. Non-peptidergic DRG neurons were identified by binding of IB4 extracted from *Griffonia simplicifolia* (BSI-B_4, FITC conjugate; Sigma-Aldrich, St. Louis, MO, USA). Coverslips were pretreated with 3 µg/mL IB4 for 5 minutes and washed for 3 minutes before beginning patching [28]. Neurons with a continuous green ring around the perimeter of the soma were considered IB4-positive.

4.2.5. Data analysis

Statistical analyses were performed using Prism v7.03 (Graphpad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± SEM or the incidence (% of neurons tested). The Shapiro-Wilk test was used to assess normality for continuous measures. Comparisons between incidence measures were made using Fisher's exact tests with Bonferroni corrections for multiple comparisons. Significance for all statistical tests was set at $P < 0.05$ and all reported P values are two-tailed.

4.3. Results

4.3.1. Probable nociceptors exhibit 2 predominant electrophysiological types *in vitro*: rapidly accommodating and nonaccommodating

Two distinct types of neurons were observed, “Nonaccommodating” (NA, **Fig. 12A**) and “Rapidly Accommodating” (RA, **Fig. 12B**), that exhibited opposite electrophysiological response patterns to ascending series of 2 second depolarizing steps delivered when a neuron was held under current clamp at an initial membrane potential of -60 mV. The NA type represented 69% of sampled neurons. Characteristic features of NA neurons were a relatively low rheobase and repetitive firing in response to injecting current equal to 2X rheobase (**Fig. 12A** and **Table 4**). An unusual feature was the random latency to the first AP at rheobase, which could occur at any time during the 2 second step depolarization. This is evident in the ranked distribution of first AP latencies, which appear evenly distributed and form a nearly straight line from shortest to longest latency (**Fig. 12C**). Some NA neurons at rheobase (**Fig. 12C**) and most neurons at 2X rheobase (**Fig. 12D**) fired multiple irregularly spaced APs during the depolarizing steps (**Table 4**). All tested NA neurons fired multiple APs to one or more of the steps between 1X and 2X rheobase, although not to all suprathreshold steps. In each NA neuron, these irregularly occurring APs were equally likely to occur at any time after the first AP during repetitive firing, as shown in the raster plots (**Figs. 12C, D**). The lack of any tendency for the interspike interval to increase during repetitive firing confirmed the lack of AP accommodation (**Fig. 12E**). This irregular NA activity continued for as long as the neurons were depolarized (>60 seconds, data not shown).

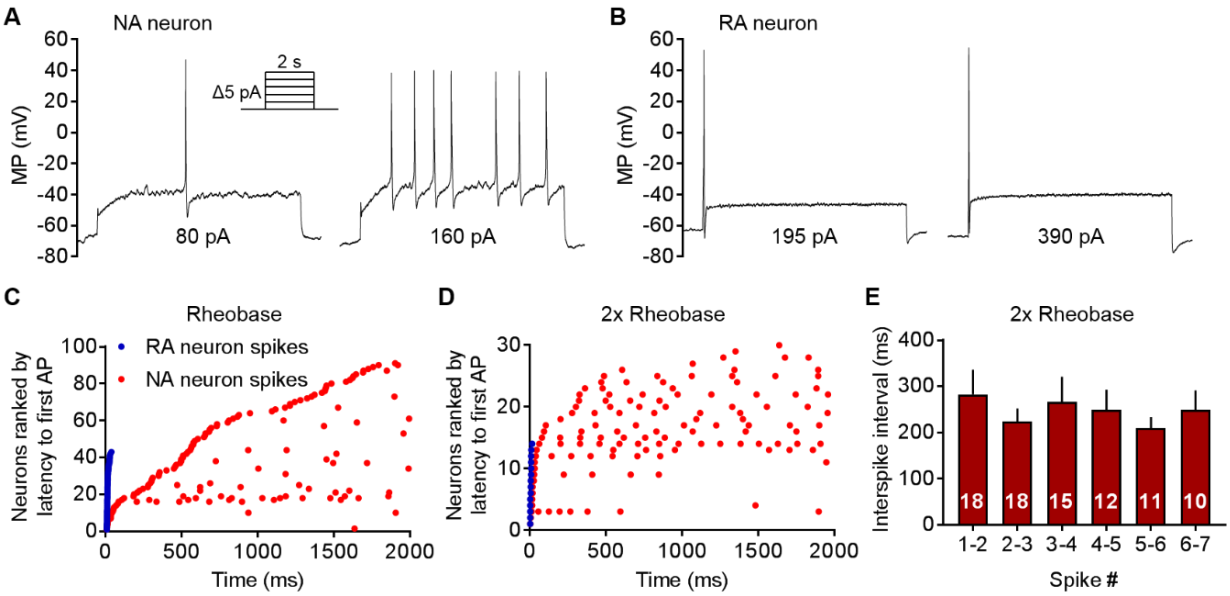


Figure 12. Two electrophysiologically distinct types of nociceptors exhibit opposite response patterns to prolonged depolarization. DRG neurons from naive rats (n = 18) were sampled using whole-cell recordings 18 to 30 hours after dissociation. **(A)** Representative AP discharge at rheobase and 2X rheobase in an NA neuron. An ascending series of 2 second depolarizing current steps was injected in 5 pA increments at 4 second intervals. A constant holding current that initially set membrane potential to -60 mV was continuously injected throughout the sequence. **(B)** Typical discharge in an RA neuron during the same test protocol. **(C)** Distribution of first AP latencies (ranked from shortest to longest) and time of occurrence of additional APs at rheobase across the 2 second depolarizing step in 95 NA neurons (initial APs are leftmost red dots) and 43 RA neurons (APs are blue dots). Additional activity is indicated along the same row at the time of each AP (red dots) for each repetitively firing neuron. **(D)** Timing of APs in the same tests at 2X rheobase from the subsets of neurons in which the depolarizing steps reached this level (30 NA neurons and 14 RA neurons). Each row represents a single neuron. **(E)** Interspike intervals at 2X rheobase in NA neurons fired ≥2 APs. Bars show the mean ± SEM, numbers in bars show neuronal sample size. AP, action potential; DRG, dorsal root ganglion; MP, membrane potential; NA, nonaccommodating; RA, rapidly accommodating. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe, Jinbin Tian, and Elia R. Lopez.

In stark contrast, the RA type (31% of sampled neurons) never fired more than a single AP in these tests, which always occurred at the onset of the step depolarization (**Figs. 12B-D**). Interestingly, within the stimulation range of 1 to 3X rheobase, no RA neurons responded with multiple APs. Only at very high stimulus currents, did some RA neurons fire a brief burst of 2 or 3 APs (not shown), and these were always confined to the onset of the stimulus. Compared to NA neurons, RA neurons showed a significantly more hyperpolarized RMP, higher rheobase, much shorter latency to the first AP, and lower membrane time constant (**Table 4**). Individual APs and AHPs evoked by 2 ms depolarizing pulses were similar between NA and RA neurons. The only statistically significant difference found in these samples was for AP duration at half-amplitude to be ~20% briefer in the NA neurons than in RA neurons (**Table 4**). No significant differences were found between NA and RA neurons in soma diameter or membrane capacitance (**Table 4**). Interestingly, far greater excitability was found in NA neurons than in RA neurons, despite the NA neurons being more hyperpolarized after each of the larger depolarizing steps in the rheobase/repetitive firing test sequence. This is illustrated in **Figures 12A** and **12B**. Although both neurons had the same -60 mV holding potential at the beginning of the series of depolarizing steps (not shown), membrane potential at the beginning of later steps in the series was more negative in NA neurons than in RA neurons; in the illustrated NA neuron, this potential was ~-70 vs ~-65 mV in the RA neuron when rheobase and 2X rheobase were reached. This residual post-depolarization hyperpolarization resulted from there being insufficient time (2 seconds) between the larger 2 second depolarizing steps for recovery of membrane potential to -60 mV (a trade-off to allow for numerous tests on each neuron).

Properties of NA and RA neurons				
Property	**NA neurons**	**RA neurons**	**Significance, *P***	**Test**
RMP (mV)	-62.1 ± 0.9 (96)	-65.2 ± 1.1 (43)	< 0.05	UPT
Rheobase (pA)	88.3 ± 7.0 (96)	169.2 ± 9.4 (43)	< 0.0001	MW
AP latency at rheobase (ms)	812.6 ± 82.1 (96)	14.6 ± 1.1 (43)	< 0.0001	MW
Number of APs at rheobase	1.6 ± 0.1 (96)	1.0 ± 0.0 (43)	< 0.0001	MW
Number of APs at 2x rheobase	3.4 ± 0.6 (32)	1.0 ± 0.0 (14)	< 0.0001	MW
Membrane time constant (τ, ms)	16.5 ± 1.8 (67)	5.4 ± 0.5 (25)	< 0.0001	MW
Membrane resistance (MΩ)	355.8 ± 24.8 (95)	333.6 ± 29.6 (43)	0.95	MW
Membrane capacitance (pF)	28.5 ± 0.9 (96)	29.8 ± 1.0 (43)	0.31	MW
Soma diameter (μm)	24.0 ± 0.5 (51)	25.5 ± 0.5 (24)	0.07	MW
AP half-amplitude duration (ms)	2.7 ± 0.3 (12)	3.3 ± 0.2 (7)	< 0.05	MW
Capsaicin sensitivity (%)	70% (43/61)	67% (20/30)	0.81	F
IB4 Binding (%)	49% (17/35)	73% (8/11)	0.19	F

Table 4. Properties of NA and RA neurons**.** Data were collected from DRG neurons taken from naive rats (n = 18) 18 to 30 hours after dissociation. Sensitivity to 1 μM capsaicin was tested in neurons from 7 rats, and binding of IB4 was tested in neurons from 4 rats. Each value is the mean ± SEM followed in parentheses by number of neurons tested. Tests: UPT, unpaired t test; MW, Mann-Whitney U; F, Fisher exact test. AP, action potential; DRG, dorsal root ganglion; NA, nonaccommodating; RA, rapidly accommodating; RMP, resting membrane potential. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared the table; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe, Jinbin Tian, and Elia R. Lopez.

4.3.2. Only nonaccommodating neurons exhibit ongoing activity when perfused with capsaicin or depolarized with injected current

Evidence that many of the NA and RA neurons are nociceptors was obtained by testing capsaicin sensitivity and binding of IB4. A majority of NA neurons and RA neurons tested with 1 μM capsaicin responded strongly under current clamp (**Fig. 13**) or voltage clamp (not shown; see [377]), indicating that large fractions of both types are TRPV1-expressing nociceptors (**Table 4**). In addition, about half of the sampled NA neurons and 3 quarters of the RA neurons bound IB4, suggesting that both types contain large fractions of non-peptidergic nociceptors

(**Table 4**). Although most small dissociated neurons and nearly all capsaicin-sensitive and IB4-binding neurons in rats are likely to be nociceptors [117,132,211,282,283,329], a minority of small DRG neurons are not nociceptive [100,117] and most small DRG neurons were not tested for capsaicin sensitivity or IB4 binding in this study. Thus, individual neurons selected for study were considered probable nociceptors, with the caveat that a minority of tested neurons would not have been nociceptive. Given the high incidence of capsaicin sensitivity and/or IB4 binding in NA and RA neurons, general properties established across sufficiently large samples of small DRG neurons under the culture conditions described primarily represent the properties of small nociceptors, and that these include 2 physiologically defined classes, NA and RA.

The responses of each electrophysiological type to capsaicin under current clamp provided additional evidence that NA neurons but not RA neurons are capable of OA. Perfusion of capsaicin (1 μM) evoked multiple APs in NA neurons under current clamp (**Fig. 13A**) (see also [377]), whereas none of the tested RA neurons (n = 5 from 3 rats) discharged any APs despite similar depolarization by capsaicin treatment (**Fig. 13B**). A low concentration of capsaicin (10 nM) can sometimes activate isolated small DRG neurons while depolarizing the neurons to between -50 and -45 mV [377]. To see whether similar depolarization can produce OA such as that produced by capsaicin, a 30 second step depolarization to -45 mV was produced by injecting current through the patch pipette. This evoked OA in 30% of the NA neurons but in none of the RA neurons (**Fig. 13C**).

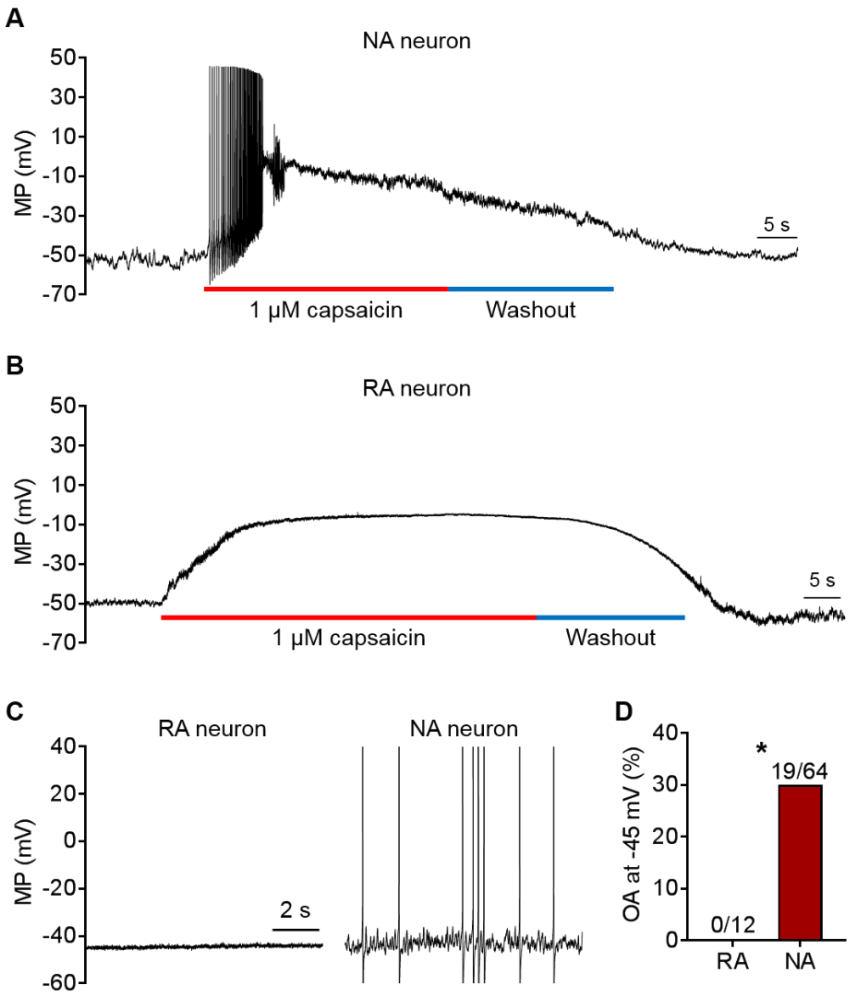


Figure 13. Nonaccommodating and RA neurons are depolarized to a similar degree by superfusion of capsaicin, but only NA neurons exhibit repetitive discharge when depolarized by a high dose of capsaicin or by injected current that mimics depolarization produced by a low dose of capsaicin. **(A)** Representative example of depolarization and discharge evoked by a high dose of capsaicin (1 μM) in an NA neuron. **(B)** Example of depolarization evoked by the same dose of capsaicin in an RA neuron. Notice the lack of APs. **(C)** Examples showing part of the responses to prolonged depolarization (30 seconds) to -45 mV in RA and NA neurons similar to that produced by 10-nM capsaicin (see text). **(D)** OA was promoted in NA neurons but not RA neurons by artificial depolarization to -45 mV. **P* < 0.05, the Fisher exact test. Neurons are from a subset (n = 12) of the naive rats used for **Figure 12**. AP, action potential; MP, membrane potential; NA, nonaccommodating; OA, ongoing activity; RA, rapidly accommodating. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe and Jinbin Tian.

Study 2: *Three functional aspects of membrane potential synergistically promote ongoing activity in nonaccommodating neurons following spinal cord injury*

4.4. Rationale

In principle, there are 3 functional aspects of membrane potential that could facilitate OA: 1) depolarization of RMP, 2) hyperpolarization of the voltage threshold for AP generation, and 3) enhancement of transient depolarizing spontaneous fluctuations (DSFs) that trigger APs. Contusive SCI in rats induces persistent OA generated peripherally in primary nociceptors in a peripheral skin-nerve preparation [57] and within the DRG in probable C-fiber and Aδ nociceptors *in vivo* [28]. It also dramatically enhances SA in small DRG neurons (primarily nociceptors) *in vitro* [25,28,377,389]. While the signaling mechanisms important for the maintenance of SCI-induced SA are beginning to be elucidated [25], it is not fully known how the 3 functional aspects of membrane potential facilitate sustained OA in putative nociceptors. Given that the NA neurons are specialized for generating OA, it is likely that SCI-induced SA is unique to the NA neurons and does not occur in RA neurons. In addition, enhanced evoked pain and avoidance of noxious probes in the MC test (*Chapter 2*) may be due to increased excitability in response to extrinsic depolarizing stimuli and/or increased SA in NA neurons that might maintain central sensitization. Indeed, C-fiber SA and robust increases in AP firing rates are both observed *in vivo* following deep tissue incisions [386]. To address these predictions, small DRG neurons dissociated from naïve, sham, and SCI rats at 1-6 months post-injury were tested for SA and other measures of increased excitability, and electrophysiologically profiled as NA and RA neurons.

4.5. Additional materials and methods

4.5.1. Spinal cord injury surgical procedure

Surgeries were performed at McGovern Medical School as previously described [25,28,377,389]. One of two methods of anesthesia were used: intraperitoneal (i.p.) injection of ketamine (60 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg) or isoflurane (induction 4-5%; maintenance 1-2%). Rats were determined to be deeply anesthetized and areflexic before proceeding. Local anesthetic (bupivacaine, 2 mg/kg) was delivered subcutaneously (s.c.) at the incision site near T10 before incising the skin from T8-T12. Laminectomy of the T10 vertebrae was followed by contusion impact (150 kdyne, 1 s dwell time) using an Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation, LLC, Fairfax Station, VA, USA). Following impact, the paravertebral muscles were closed with vicryl-coated, absorbable suture and the skin incision was closed with 9 mm wound clips. Sham rats received the same laminectomy surgery minus spinal impact. Rats were returned to their home cage and placed on a heating pad maintained at 37°C. The analgesic buprenorphine hydrochloride (0.02 mg/kg; Buprenex, Reckitt Benckiser Healthcare Ltd., Hull, England, UK) was administered in 0.9% saline (2 mL/kg, i.p.) twice, daily up to 5 days post-surgery. The prophylactic antibiotic enrofloxacin (0.3 mL; Enroflox, Norbrook, Inc., Overland Park, KS, USA), was also administered in 0.9% saline daily up to 10 days post-surgery. Manual bladder evacuations were performed twice, daily until rats recovered neurogenic bladder voiding. The day after surgery hindlimb locomotion was assessed using the BBB score [24]. Only sham rats with BBB scores of 21 for both hindlimbs were accepted. The majority of SCI rats were scored a 0 or 1 for both hindlimbs 1 day after surgery. Naïve rats were transported to the surgical suite at the same time as surgeries were performed, but were otherwise left undisturbed in their home cages.

4.5.2. Quantifying spontaneous fluctuations of membrane potential and action potential threshold

Published methods for quantifying spontaneous fluctuations (SFs; first known descriptions in C-type DRG neurons [239,337]) of membrane potential in DRG neurons rely on power spectral density analyses, which require that the SFs be oscillations or appear at regular intervals if not oscillatory [8–10,224,381,383]. We developed a novel series of algorithms that identifies waveforms independent of frequency or regularity, inspired by the Ramer-Douglas-Peucker algorithm [107,294] to identify curves, in order to quantify the irregular DSFs observed in whole-cell recordings. Our program, termed SFA.py, was written and tested using Python v3.5.2 (Python Software Foundation, Beaverton, OR) and Anaconda v4.1.1 (Continuum Analytics, Austin, TX) with dependency on matplotlib and NumPy libraries. Time and voltage coordinate data for 30-50 second periods exported from PatchMaster were imported into the script. SFA.py then performed the following functions: 1) generate a linear regression model as an initial estimate of membrane potential; 2) group the runs of unidirectional residuals into discrete membrane fluctuations, and employ user-defined criteria to classify some of these as AP/AHP complexes; 3) exclude AP/AHPs from analysis, then calculate the RMP at each point as a sliding median of the raw data within a 1 second window centered on that point – this accounts for slow, non-linear changes in RMP which would otherwise increase or decrease the estimated amplitude of a given fluctuation; 4) run the groups of unidirectional residuals as discrete fluctuations, then apply user-defined criteria for minimum amplitude and duration (1.5 mV and 10 ms for this study) to identify DSFs or hyperpolarizing spontaneous fluctuations (HSFs); 5) quantify and report the following values: coordinates, amplitudes, and durations of identified APs, AHPs, DSFs, and HSFs. DSFs and HSFs ≥1.5 mV were measured as differences from the sliding median of membrane potential. DSFs were subdivided into small (>1.5 to ≤3 mV), medium-sized (3-5 mV, almost always subthreshold) and large (>5 mV, often suprathreshold) DSFs as described. All HSFs were ≥1.5 mV, and were not subdivided for

further analysis. Descriptive data for the recordings also include standard deviation of the membrane potential, number of APs, AP frequency, number of DSFs and HSFs, and their frequencies. Color-coded line graphs with labeled APs, AHPs, DSFs, and HSFs were generated using the matplotlib library. Inspection of SA indicated that most APs in NA neurons were triggered by suprathreshold DSFs. As a conservative estimate of the amplitude of suprathreshold DSFs, these were assigned an amplitude equal to the AP voltage threshold. This threshold was estimated for each neuron by three independent measures that together provided a more accurate estimate of AP threshold than commonly utilized analytic methods [318] that were tested. To estimate threshold, 1) the inflection point for apparent acceleration of the change in membrane potential was measured at the base of the ascending limb of the AP, 2) the peak membrane potential was measured for the maximum subthreshold DSF found anywhere in the 1-2 second step depolarizations used to determine rheobase in the same neuron, and 3) the peak membrane potential was measured for the largest subthreshold DSF during recorded SA at RMP. The most depolarized of these three independent measurements was defined as the AP threshold for that neuron, and in all cases at least two of these three values were in good agreement with each other (within ~2 mV).

4.5.3. Data analysis

Planned comparisons between naïve, sham, and injury groups were made using 1-way ANOVA or Kruskal-Wallis tests followed by Tukey's or Dunn's post-hoc tests. To assess main injury-related effects, electrophysiological measures collected from neurons recorded across multiple experiments (e.g., days, surgeons) were pooled according to rat surgical history: naïve, sham, or SCI. To assess SA-dependent effects, neurons were pooled based on the presence or absence of SA and the aforementioned analyses were performed.

4.6. Results

4.6.1. Spinal cord injury increases spontaneous activity in nonaccommodating but not rapidly accommodating neurons

SA was found in at least some of the NA neurons taken from naive, sham, or SCI rats, but was not found in any RA neurons (**Figs. 14A** and **14B**). As predicted by earlier findings [25,28,376,377,389], the incidence of SA was significantly greater in neurons from SCI rats than in neurons from naive or sham rats (**Fig. 14B**). In contrast to an earlier finding [28], the incidence of SA in the sham group was modestly but significantly higher than in the naïve group (**Fig. 14B**). This finding and other evidence for persistent hyperexcitability in the sham group (see below and **Table 5**) differ from an earlier study [28]. Unmasking of sham effects may reflect improvements in DRG extraction and dissociation procedures that reduced cellular stress, lowering the incidence of SA in the naive group in this study. These results indicate that SCI strongly enhances SA in NA neurons but not in RA neurons. In addition, tissue injury caused by the sham surgery can produce a small increase in incidence of SA in NA neurons. One possibility that cannot be ruled out is that extrinsic factors in the neuronal cultures (either soluble factors or contact signals from small adjacent or underlying cells invisible to the microscopy methods used) might contribute to the neuronal activity observed after SCI, but it is highly likely that SA is produced by mechanisms intrinsic to NA neurons. No differences are observed in the incidence of SCI-induced SA in cultures across a wide range of cell densities, whether the nearest neighboring cell is several hundreds of microns away or in clear contact with the sampled neuron, and because the incidence of SA is unchanged by rapid perfusion or no perfusion of the culture dish [25,28,376,377] (and unpublished observations).

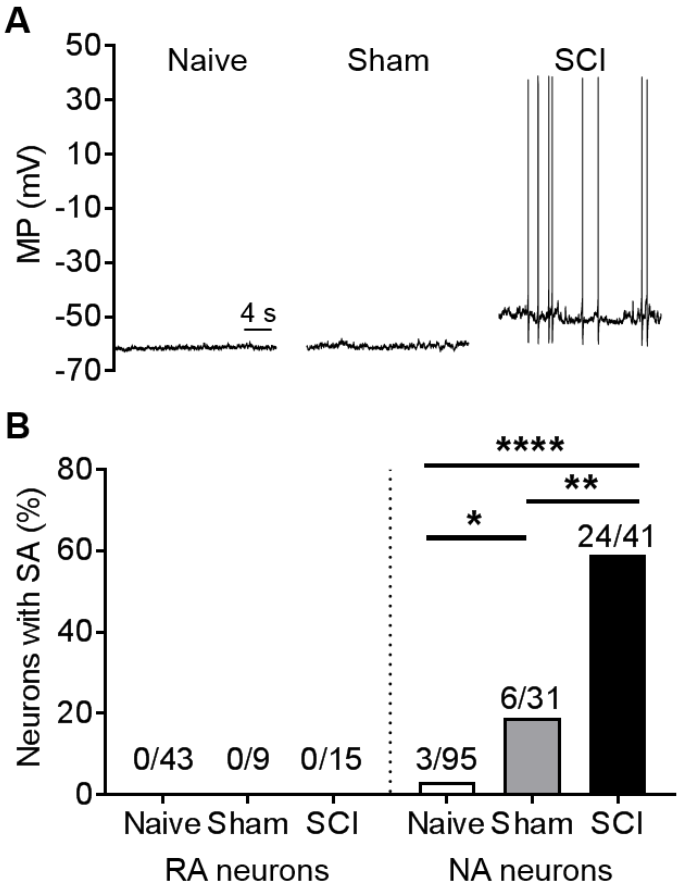


Figure 14. Injury-induced SA occurs in NA neurons but not RA neurons. Small DRG neurons from naive (n = 18), sham (n = 5), and SCI rats (n = 13) were recorded 18 to 30 hours after dissociation under current clamp without injected current for ≥1 minutes to measure SA. **(A)** Representative recordings of NA neurons from the indicated groups. **(B)** SA incidence in RA and NA neurons in each group. Fractions represent number of neurons with SA/total sample. Comparisons made using the Fisher exact test (Bonferroni corrected), *P < 0.05, **P < 0.01, ****P < 0.0001. DRG, dorsal root ganglion; MP, membrane potential; NA, nonaccommodating; RA, rapidly accommodating; SA, spontaneous activity; SCI, spinal cord injury. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe.

Table 5. Effects of SCI on NA and RA neuron excitability. Data were collected 18 to 30 hours after dissociation of DRG neurons taken from naive (n = 18), sham (n = 5), or SCI rats (n = 13). Comparisons were not made between groups for RA neurons in the cases of number of APs at rheobase or at 2X rheobase because repetitive firing did not occur in any RA neuron. Each value is the mean ± SEM followed in parentheses by number of neurons tested. Tests: KW, Kruskal-Wallis followed by Dunn tests; ANOVA, 1-way ANOVA followed by Tukey tests. ANOVA, analysis of variance; AP, action potential; DRG, dorsal root ganglion; NA, nonaccommodating; RA, rapidly accommodating; RMP, resting membrane potential; SCI, spinal cord injury. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared the table; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe.

Summary of SCI-induced alterations in excitability in NA and RA neurons							
Property	**Naive**	**Sham**	**SCI**	**Naïve vs sham, *P***	**Naïve vs SCI, *P***	**Sham vs SCI, *P***	**Test**
NA neurons							
RMP (mV)	-61.9 ± 0.8 (95)	-60.1 ± 1.7 (31)	-54.2 ± 1.5 (41)	0.99	< 0.001	< 0.05	KW
AP voltage threshold (mV)	-34.0 ± 0.5 (94)	-33.6 ± 1.4 (31)	-37.1 ± 1.0 (39)	0.94	< 0.05	< 0.05	ANOVA
Rheobase (pA)	89.1 ± 7.0 (95)	56.6 ± 9.6 (31)	44.9 ± 7.0 (41)	< 0.05	< 0.001	0.99	KW
AP latency at rheobase (ms)	803.3 ± 82.5 (95)	616.3 ± 94.4 (31)	305.3 ± 48.3 (41)	0.99	< 0.001	< 0.05	KW
Number of APs at rheobase	1.6 ± 0.1 (95)	1.6 ± 0.3 (31)	2.6 ± 0.4 (41)	0.99	0.14	0.23	KW
Number of APs at 2x rheobase	4.0 ± 0.6 (32)	4.1 ± 0.7 (20)	6.5 ± 0.9 (22)	0.99	< 0.05	0.17	KW
Membrane resistance (MΩ)	355.8 ± 24.8 (95)	480.4 ± 52.6 (31)	464.5 ± 30.9 (41)	< 0.05	< 0.01	0.99	KW
RA neurons							
RMP (mV)	-65.2 ± 1.1 (43)	-64.9 ± 1.1 (9)	-61.8 ± 2.1 (15)	0.99	0.25	0.55	ANOVA
AP voltage threshold (mV)	-28.4 ± 0.9 (42)	-26.1 ± 2.0 (9)	-31.0 ± 1.6 (15)	0.49	0.57	0.25	ANOVA
Rheobase (pA)	169.2 ± 9.4 (43)	143.9 ± 17.3 (9)	124.7 ± 22.0 (15)	0.98	0.06	0.99	KW
AP latency at rheobase (ms)	14.6 ± 1.1 (43)	14.7 ± 2.3 (9)	24.4 ± 4.4 (15)	0.99	0.10	0.93	KW
Number of APs at rheobase	1.0 ± 0.0 (43)	1.0 ± 0.0 (9)	1.0 ± 0.0 (15)	-	-	-	-
Number of APs at 2x rheobase	1.0 ± 0.0 (14)	0.8 ± 0.2 (5)	1.0 ± 0.0 (10)	-	-	-	-
Membrane resistance (MΩ)	333.6 ± 29.6 (43)	785.8 ± 146.3 (7)	295.9 ± 39.0 (14)	< 0.01	0.99	< 0.01	KW

SCI-induced SA in dissociated small DRG neurons has been found up to 8 months after SCI [28] but previous studies did not distinguish NA from RA neurons. The incidence of SCI-induced SA in neurons from rats tested 1 to 3 months and 3 to 6 months after SCI was compared to see whether the occurrence of SA in NA neurons changed over the period of this study. The mean incidence of NA neurons with SA at 1 to 3 months (63 ± 7%, n = 6 rats) was not significantly different from the incidence at 3 to 6 months (70 ± 3%, n = 5 rats) (P = 0.36, unpaired t test). The ratio of NA to RA neurons between groups was compared to determine whether SCI might shift one type of probable nociceptor (RA or NA) into the other type. Very little difference was found in the ratio of NA to RA neurons in the naïve or sham groups, so these were combined into a single control group. In this control group, 71% of 143 tested neurons were NA and 29% were RA. In the SCI group, 77% of 198 tested neurons were NA and 23% were RA. The small shift from RA to NA was not statistically significant (P = 0.098), but the possible trend suggests that further investigation is warranted into the question of whether *in vivo* injury or inflammation might promote a transition of one nociceptor type into the other. The NA/RA ratio was not affected by time after SCI (82 ± 7% NA neurons at 1-3 months, n = 6 rats; 84 ± 5% NA neurons at 3-6 months, n = 5 rats; P = 0.86, unpaired t test).

4.6.2. Spinal cord injury persistently depolarizes resting membrane potential and lowers action potential threshold in nonaccommodating neurons

What are the neurophysiological mechanisms by which SCI promotes SA and OA in NA neurons? Two of the 3 intrinsic functional aspects of membrane potential that in principle can generate SA (and promote extrinsically driven OA) are prolonged depolarization of RMP and a hyperpolarizing shift in the voltage threshold for AP generation. Persistent SCI-induced depolarization of RMP was found previously in dissociated small DRG neurons [28], but AP voltage threshold was not measured, and whether either of these SA-promoting effects occurs in NA neurons after SCI has not been documented. Compared to NA neurons in the naive and sham groups, NA neurons in the SCI group showed significant depolarization of RMP and

significant reduction in voltage threshold for AP generation (**Table 5**). No significant differences in these properties were found between the naive and sham groups. Three other measures also revealed significantly greater excitability in NA neurons in the SCI group vs the naive group: rheobase dropped by 50%, repetitive firing in response to currents twice the rheobase value nearly doubled, and membrane resistance increased by 30% (**Table 5**). Interestingly, rheobase and membrane resistance in the sham group were significantly different from values in the naive group, providing additional evidence for persistent hyperexcitability after sham surgery. No significant effects of SCI were found in RA neurons (**Table 5**). Fewer RA than NA neurons were examined, so it is possible that weak effects of SCI or sham treatment could be revealed by larger samples of RA neurons. These results show that 2 physiological alterations important for driving SA, persistent depolarization and reduction of AP voltage threshold, are induced in NA neurons by SCI. All the measures of hyperexcitability were especially prominent in spontaneously active NA neurons taken from SCI rats (**Table 6**), consistent with a hyperexcitable state being induced by SCI that functions to promote SA [28]. In addition, sham surgery can also persistently increase excitability of NA neurons, expressed as lowered rheobase, but without substantial alteration of RMP or AP voltage threshold.

Comparison of silent and spontaneously active NA neurons taken from SCI rats				
Property	**Silent**	**Exhibiting SA**	**Significance, *P***	**Test**
RMP (mV)	-61.6 ± 1.8 (17)	-49.0 ± 1.5 (24)	< 0.0001	MW
AP voltage threshold (mV)	-34.1 ± 1.0 (17)	-39.3 ± 1.5 (22)	< 0.05	UPT
Rheobase (pA)	80.3 ± 11.1 (17)	19.8 ± 4.4 (24)	< 0.0001	MW
AP latency at rheobase (ms)	517.8 ± 84.4 (17)	154.8 ± 32.2 (24)	< 0.001	MW
Number of APs at rheobase	1.3 ± 0.2 (17)	3.5 ± 0.6 (24)	< 0.01	MW
Number of APs at 2x rheobase	3.4 ± 0.5 (5)	7.5 ± 1.1 (17)	0.07	UPT
Membrane resistance (MΩ)	411.3 ± 47.8 (17)	502.3 ± 39.5 (24)	0.10	MW

Table 6. Properties of silent and spontaneously active NA neurons taken from SCI rats. Data were collected from a randomly selected subset of the SCI group in Table 2 (n = 8 rats). Each value is the mean ± SEM followed in parentheses by number of neurons tested. Tests: UPT, unpaired t test; MW, Mann-Whitney U. AP, action potential; NA, nonaccommodating; RMP, resting membrane potential; SA, spontaneous activity; SCI, spinal cord injury. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared the table; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe.

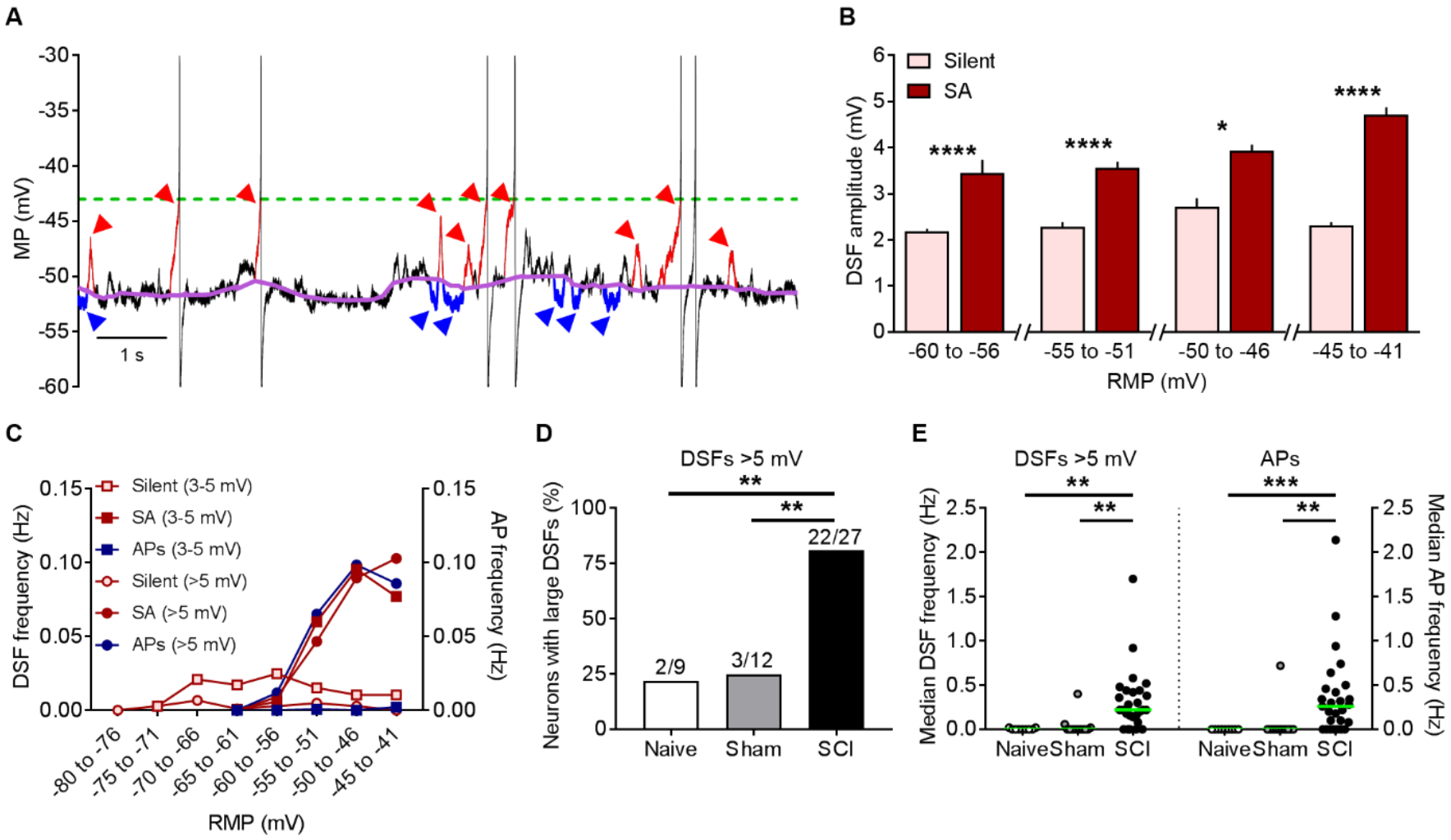
4.6.3. Spinal cord injury persistently enhances depolarizing spontaneous fluctuations in nonaccommodating neurons

The third functional aspect of membrane potential that in principle can generate SA and promote extrinsically driven OA is an increase in the frequency of large DSFs that can reach AP threshold. Irregular SFs of membrane potential have long been evident in published whole-cell patch recordings from dissociated small- and medium-sized DRG neurons, but they have received remarkably little experimental attention. The most detailed study [337] found no obvious association between fluctuation amplitude and SA in a rat CCI model of neuropathic pain, but systematic quantitative measurements were not performed. Two quantitative approaches were used to test whether SCI increases DSF amplitude and frequency in NA neurons. The first approach was to measure total fluctuation amplitude (peak to peak) after SCI. Preliminary results (not shown) indicated that, unlike the regular sinusoidal oscillations in large and medium-sized DRG neurons that are enhanced by axotomy [7,9,223], the irregular fluctuations in small DRG neurons lack large sinusoidal components that contribute significantly to OA generated at RMP negative to -40mV (see also [9]), which is the RMP range at which SA and OA have been investigated [25,28,377,389]. Thus, as an alternative to fast Fourier transform analysis, the SD of all points (excluding APs and AHPs) in randomly selected 50 second samples in NA cells was compared across groups. SD provides a symmetrical measure of dispersion of the fluctuations from the mean RMP. The SDs of fluctuation amplitudes were significantly larger in the SCI group (mean of the fluctuation SDs for each neuron, 3.0 ± 0.3 mV, 27 neurons) than in the naive group (1.2 ± 0.3 mV, 9 neurons) or sham group (1.1 ± 0.2 mV, 12 neurons) (Tukey multiple comparison $P < 0.01$ in each case). This result shows that SCI increases fluctuation amplitudes but does not distinguish between any differential effects of SCI on DSFs and HSFs.

Plotting all points in each trace relative to the median instead of the mean revealed a skew in the depolarizing direction, raising the possibility that SCI might selectively promote the

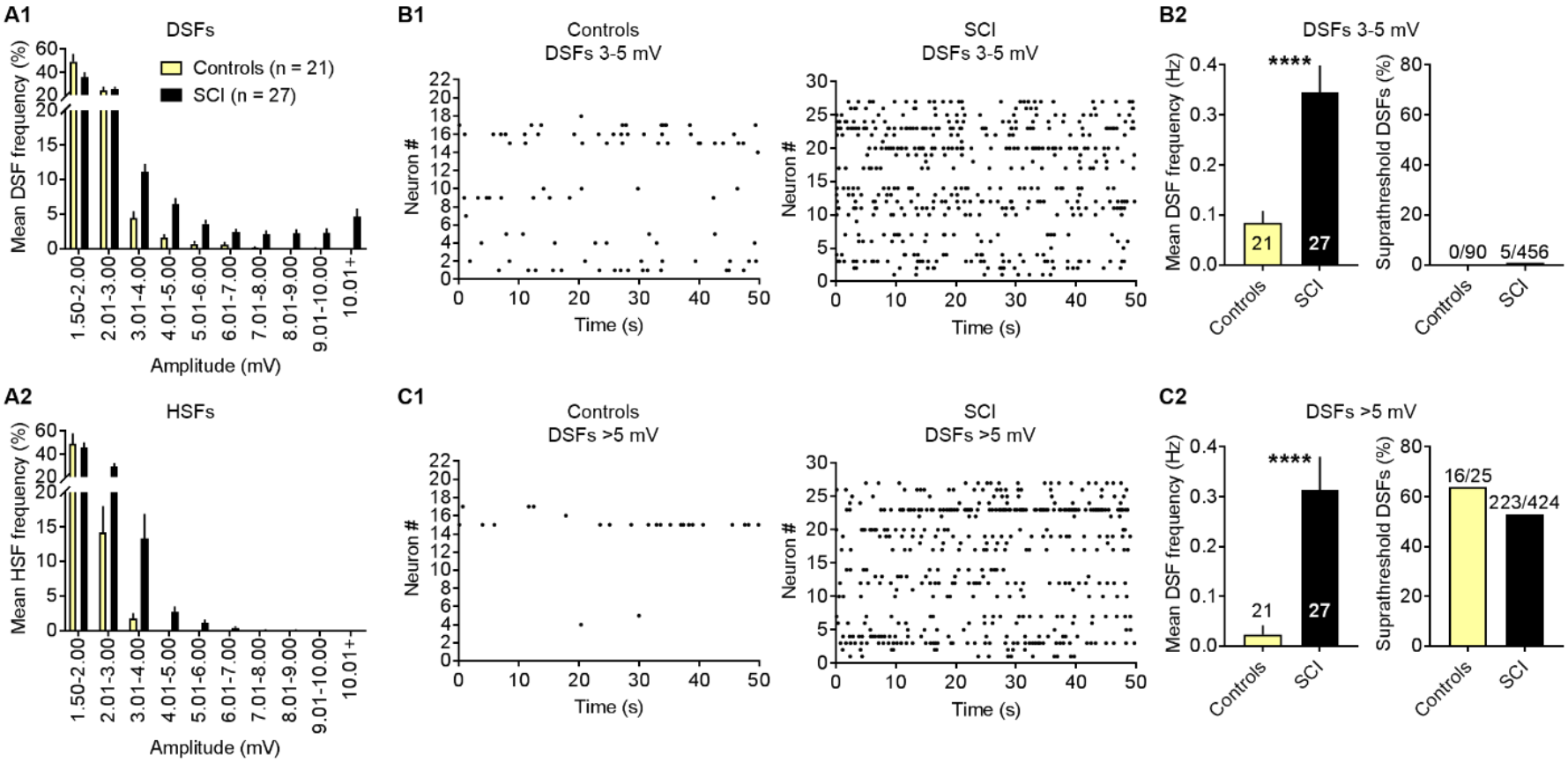
generation of large DSFs in addition to (or instead of) enhancing oscillatory or hyperpolarizing fluctuations. This is important because HSFs as well as sinusoidal oscillations have been described in isolated DRG neurons [9,239]. To rigorously test this prediction, a novel SF analysis program was used to measure DSFs and HSFs, which were defined by reference to a sliding median of all points measured during 50 second samples. An example of part of an analyzed trace is shown in **Figure 15A**. Note that DSFs are defined operationally and are unlikely to represent unitary events; indeed, there seems to be complex summation of smaller depolarizing (and possibly hyperpolarizing) events in many of the DSFs shown. Analysis of DSFs in NA neurons exhibiting SA (from naive, sham, and SCI groups) revealed that mean DSF amplitude was largest (>5 mV) when RMP was between -45 and -40 mV (**Fig. 15B**). Given that the voltage threshold for AP generation after SCI ranged from -28 to -50 mV, and RMP ranged between -70 and -40 mV (see also **Table 5**), relatively large DSFs (>5 mV) could reach AP threshold often enough to contribute significantly to observed SA. Analysis of NA neurons exhibiting SA showed that the frequency within each trace of DSFs with amplitudes >5 mV (most of which initiated APs; see below) and medium-sized DSFs with amplitudes of 3 to 5 mV (which almost never evoked APs) showed striking parallels to the frequency of APs in the same neurons plotted as a function of RMP (**Fig. 15C**). This close parallel provides strong evidence that large DSFs play an important role in triggering APs in NA neurons. Importantly, significantly more NA neurons in the SCI group had large DSFs (>5 mV) than did neurons in the naive or sham groups (**Fig. 15D**). Moreover, frequencies both of large DSFs and of APs within each recording were significantly greater in the SCI group (**Fig. 15E**).

Figure 15. Spinal cord injury enhances the amplitudes and frequencies of DSFs in NA neurons. DSFs were quantified with an algorithm that estimates RMP through a sliding median function, and then identified SFs exceeding 1.5 mV above and below this continuously changing reference line. **(A)** Sample recording of SA after SCI. Color labels: purple undulating line – sliding median, red arrowheads and red trace segments – subthreshold and suprathreshold DSFs ≥3 mV, blue arrowheads and blue trace segments – all HSFs ≥1.5 mV, and green dashed line – AP threshold. **(B)** Neurons with SA (n = 27) showed enhanced DSF amplitudes compared with silent neurons at RMPs between -60 and -40 mV. DSFs were binned according to the RMP at the DSF onset. DSF sample sizes left to right: 286, 68, 186, 386, 49, 568, 91, and 425. Data are shown as mean ± SEM. Comparisons between silent and SA groups at each bin made using Mann-Whitney U tests. **(C)** The frequency of medium amplitude DSFs (3-5 mV, squares) and large DSFs (>5 mV, circles) increased at more depolarized RMPs in neurons with SA (solid symbols) but not in silent neurons (open symbols), paralleling the increase in AP frequency (blue circles). Almost no APs were triggered by medium-sized DSFs (blue squares) in neurons with or without SA. DSFs and APs from neurons in naive, sham, and SCI conditions were pooled together into silent and SA groups for analysis. Each point represents frequency (Hz) calculated by dividing the total number of DSFs or APs by the number of neurons per group (silent n = 21, SA n = 27) and the recording duration (50 seconds for each neuron). **(D)** Large DSF incidence was significantly greater after SCI. Fractions represent number of neurons exhibiting large DSFs/total sample. Comparisons made using Bonferroni-corrected Fisher exact tests. **(E)** SCI increased the frequency of large DSFs and APs in each neuron. Green lines – medians. Overall significance assessed with the Kruskal-Wallis test, multiple comparisons with Dunn tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Neurons are a randomly selected subset taken from the naive (n = 2), sham (n = 3), and SCI rats (n = 8) used in **Figure 14**. AP, action potential; DSF, depolarizing spontaneous fluctuation; HSF, hyperpolarizing spontaneous fluctuation; MP, membrane potential; NA, nonaccommodating; RMP, resting membrane potential; SA, spontaneous activity; SCI, spinal cord injury. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments; Ryan M. Cassidy, Max A. Odem, and Edgar T. Walters designed the SF analysis program; additional experiments were performed by Alexis G. Bavencoffe.


A
MP (mV)
-30
-35
-40
-45
-50
-55
-60
1 s
B
Silent
SA
DSF amplitude (mV)
****
****
*
****
-60 to -56
-55 to -51
-50 to -46
-45 to -41
RMP (mV)
C
Silent (3-5 mV)
SA (3-5 mV)
APs (3-5 mV)
Silent (>5 mV)
SA (>5 mV)
APs (>5 mV)
DSF frequency (Hz)
AP frequency (Hz)
-80 to -76
-75 to -71
-70 to -66
-65 to -61
-60 to -56
-55 to -51
-50 to -46
-45 to -41
RMP (mV)
D
DSFs >5 mV
Neurons with large DSFs (%)
2/9
3/12
22/27
Naive
Sham
SCI
E
DSFs >5 mV
APs
Median DSF frequency (Hz)
Median AP frequency (Hz)
Naive
Sham
SCI

The effects of SCI on SFs are shown in greater detail in **Figure 16**. A frequency distribution for DSFs and for HSFs was obtained for each trace and averaged across all traces in each group. No apparent differences were found in the incidence or amplitude of either DSFs or HSFs in naive compared with sham groups, so these 2 groups were pooled into a single control group for further analysis. Compared to the combined control group, SCI increased the frequency of occurrence of larger DSFs and HSFs, but most of the SCI effect on DSFs was on amplitudes from 3 to >10 mV (**Fig. 16A1**), whereas most of the effect on HSFs was on amplitudes between 2 and 4 mV (**Fig. 16A2**). Raster plots showed higher frequencies of medium amplitude (3-5 mV) (**Figs. 16B1** and **16B2**) and large DSFs (>5 mV) (**Figs. 16C1** and **16C2**) in neurons from the SCI group compared with the control group. Almost none of the 3 to 5 mV DSFs triggered APs in neurons from the SCI or control groups (**Fig. 16B2**), whereas more than 50% of DSFs >5 mV triggered APs in neurons from SCI and control groups (**Fig. 16C2**).

Figure 16. Spinal cord injury enhances the incidence of large and medium amplitude DSFs and medium amplitude HSFs in NA neurons. SCI induced a rightward shift in the frequency distribution (% of total) of DSFs **(A1)** and HSFs **(A2)** of different amplitudes. Distributions obtained from each neuron for a 50 second period were averaged across neurons; bars represent the mean ± SEM for each amplitude bin. Naive and sham groups were pooled together into a combined control group. **(B1, C1)** Medium amplitude and large DSFs showed stochastic occurrence in control and SCI neurons. Each row represents one neuron and each dot a single DSF. **(B2, C2)** SCI increased the mean frequency of medium amplitude and large DSFs in the 50 second samples, but not the fraction of large DSFs that evoked APs. Bars represent the mean ± SEM or fraction of the total sample. Significance tested with Mann-Whitney U or Fisher exact tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Neurons are the same as in **Figure 15**. AP, action potential; DSF, depolarizing spontaneous fluctuation; HSF, hyperpolarizing spontaneous fluctuation; NA, nonaccommodating; SCI, spinal cord injury. **Contributions:** Max A. Odem designed/performed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments; additional experiments were performed by Alexis G. Bavencoffe.


A1
DSFs
Controls (n = 21)
SCI (n = 27)
Mean DSF frequency (%)
1.50-2.00
2.01-3.00
3.01-4.00
4.01-5.00
5.01-6.00
6.01-7.00
7.01-8.00
8.01-9.00
9.01-10.00
10.01+
Amplitude (mV)
A2
HSFs
Mean HSF frequency (%)
Amplitude (mV)
B1
Controls
DSFs 3-5 mV
SCI
DSFs 3-5 mV
Neuron #
Time (s)
B2
DSFs 3-5 mV
Mean DSF frequency (Hz)
****
21
27
Controls
SCI
Suprathreshold DSFs (%)
0/90
5/456
C1
Controls
DSFs >5 mV
SCI
DSFs >5 mV
C2
DSFs >5 mV
16/25
223/424

Large DSFs, similar to OA observed *in vitro* and *in vivo* in presumptive C-fiber nociceptors after SCI [28], occur randomly (**Fig. 16C1**) (see also [337]). Stochastic DSF occurrence was also seen during the 2 second depolarizations used to measure rheobase and repetitive firing (**Fig. 12**). A striking finding was the much longer latency to the first AP generated in the rheobase tests in NA compared with RA neurons (**Table 4**). This is consistent with the AP at rheobase in NA neurons being triggered by infrequent, randomly occurring, large DSFs. If so, the increase in frequency of large DSFs after SCI should increase the likelihood that large DSFs occur early during depolarizing test pulses, and this should decrease the latency to the first AP. Confirming this prediction, the mean latency to the first AP generated in NA neurons during rheobase measurement in the SCI group was much shorter than the latency in the naive or sham groups (**Table 5**). Together, these findings show 1) that DSFs play a major role in generating the irregular SA found in NA neurons, and 2) that enhancement of DSF amplitude and large DSF frequency contributes to SCI-induced SA.

Study 3: *An inflammatory mediator, serotonin, acutely potentiates ongoing activity in nonaccommodating neurons from naïve rats*

4.7. Rationale

Is enhancement of DSFs and the consequent promotion of nociceptor activity solely a long-term phenomenon, perhaps unique to SCI, or can NA nociceptor DSFs also be enhanced acutely by extrinsic signals? In particular, could acute exposure to an inflammatory signal enhance DSFs and promote OA? The inflammatory mediator, serotonin, can induce pain and hyperalgesia in the periphery [2,166,222,275,313,341] (for review [230,332]). It is interesting because it has complex effects on nociceptors [189,222,308,317,343,394], one of which is to reduce AP voltage threshold [56]. In contrast to nearly all other studies of 5-HT's actions on nociceptors, which used very high 5-HT concentrations (typically 10 mM), an early study showed that 10-nM 5-HT caused alterations in TTX-resistant Na^+ current that should lower AP

threshold [133]. An implication of this observation is that a 5-HT concentration that sensitizes but does not activate NA neurons could potentiate depolarization-dependent OA in NA neurons. Potentiation of such OA would be much more likely if the same concentration of 5-HT also enhances DSFs. Dissociated DRG neurons from naïve rats were treated with 5-HT, electrophysiologically profiled as NA or RA neurons, and tested for OA in order to determine whether 5-HT had a potentiating effect on NA neurons.

4.8. Additional materials and methods: Serotonin treatment and data analysis

The day following dissociation, DRG neurons isolated from naïve rats were pretreated with 100 nM serotonin (5-hydroxytryptamine; 5-HT; Sigma-Aldrich, St. Louis, MO) dissolved in extracellular solution for 10-30 min. 5-HT remained in the recording chamber for the duration of each experiment. To assess 5-HT effects parametric t-tests and non-parametric Mann-Whitney U tests were used.

4.9. Results

4.9.1. Serotonin selectively potentiates OA in nonaccommodating neurons by lowering action potential threshold, but does not depolarize resting membrane potential

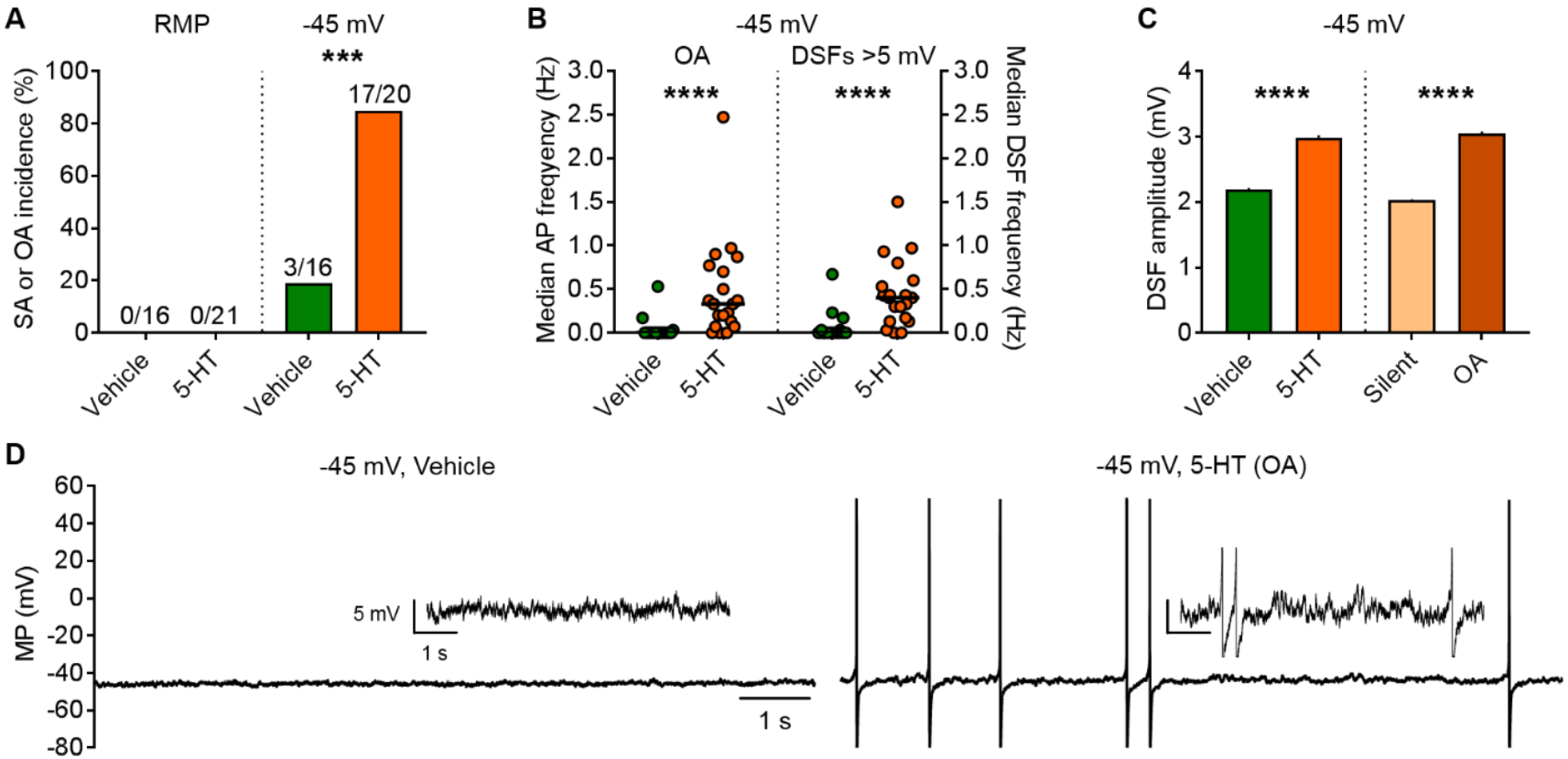
Treatment of each dish with 100 nM 5-HT for 10 to 30 minutes before and during recording produced no hint of sustained depolarization (**Table 7**). Furthermore, 5-HT did not induce OA at RMP (**Fig. 17A**, left panel). When a prolonged extrinsic depolarizing input (modeled by constant current injection through the patch pipette to hold the membrane potential at ~-45 mV for 30-60 seconds) was added to promote OA after vehicle treatment, no significant increase in the incidence of OA was found vs the incidence of SA at RMP (compare vehicle groups in left and right panels of **Fig. 17A**). By contrast, when 5-HT-treated neurons were depolarized to -45 mV, ~80% showed OA (**Fig. 17A**, right panel). At -45 mV, AP firing rates during OA and the corresponding large DSF frequencies were significantly greater in 5-

HT-treated neurons than in vehicle-treated neurons (**Fig. 17B**). Amplitudes of DSFs ≥1.5 mV were also enhanced in 5-HT-treated neurons that were depolarized to -45 mV (**Fig. 17C**, left panel), and like the effects of SCI (see **Figure 14B**), the DSFs were largest in neurons with OA (**Fig. 17C**, right panel). Examples of DSFs and APs (OA) in NA neurons held at -45 mV with and without 5-HT treatment are shown in **Figure 17D**.

Effects of 5-HT on NA and RA neurons				
Property	**Vehicle**	**5-HT**	**Significance, *P***	**Test**
NA neurons				
RMP (mV)	-67.1 ± 1.6 (16)	-66.7 ± 1.3 (21)	0.85	UPT
AP voltage threshold (mV)	-34.0 ± 1.0 (16)	-40.0 ± 1.1 (21)	< 0.001	UPT
Rheobase (pA)	92.5 ± 12.1 (16)	50.2 ± 7.8 (21)	< 0.01	UPT
AP latency at rheobase (ms)	651.5 ± 159.3 (16)	750.8 ± 135.2 (21)	0.46	MW
Number of APs at rheobase	1.1 ± 0.1 (16)	1.3 ± 0.2 (21)	0.25	MW
Number of APs at 2x rheobase	4.6 ± 1.3 (13)	5.4 ± 0.8 (21)	0.31	MW
Membrane resistance (MΩ)	350.5 ± 50.9 (16)	298.5 ± 32.5 (21)	0.66	MW
RA neurons				
RMP (mV)	-68.7 ± 2.1 (10)	-63.0 ± 3.3 (9)	0.15	UPT
AP voltage threshold (mV)	-31.4 ± 1.6 (10)	-35.7 ± 1.8 (9)	0.20	MW
Rheobase (pA)	154.0 ± 24.1 (10)	123.9 ± 26.7 (9)	0.59	MW
AP latency at rheobase (ms)	17.0 ± 3.1 (10)	18.6 ± 3.7 (9)	0.97	MW
Number of APs at rheobase	1.0 ± 0.0 (10)	1.0 ± 0.0 (9)	-	-
Number of APs at 2x rheobase	1.0 ± 0.0 (7)	1.0 ± 0.0 (7)	-	-
Membrane resistance (MΩ)	266.6 ± 37.5 (10)	269.9 ± 35.1 (9)	0.99	MW

Table 7. Effects of 5-HT on NA and RA neuron excitability. Tests were conducted in the presence of 5-HT or vehicle applied 10 to 30 minutes earlier onto small DRG neurons taken from naive rats (n = 4). Comparisons were not made between groups for RA neurons in the cases of number of APs at rheobase or at 2X rheobase because repetitive firing did not occur in any RA neuron. Tests: UPT, unpaired t test; MW, Mann-Whitney U. AP, action potential; DRG, dorsal root ganglion; NA, nonaccommodating; RA, rapidly accommodating; RMP, resting membrane potential. **Contributions:** Elia R. Lopez designed/performed experiments; Max A. Odem designed experiments, analyzed data, and prepared the table; Edgar T. Walters designed experiments.

Figure 17. Potentiation by 5-HT of OA in NA neurons. DRG neurons from naive rats (n = 4) were treated with vehicle or 100 nM 5-HT for 10 to 30 minutes before and during each recording. After measurement of any SA, extrinsically driven OA was modeled by depolarization to -45 mV under current clamp for 30 to 60 seconds. **(A)** Pretreatment with 5-HT did not induce OA at RMP but significantly increased OA at -45mV (the Fisher exact test). **(B)** In neurons tested at -45 mV, 5-HT significantly increased AP frequency during OA and large DSF frequency. Black lines – medians. Comparisons made using Mann-Whitney U tests. **(C)** 5-HT increased the amplitude of DSFs measured at -45 mV, and the neurons with OA showed larger DSFs than silent neurons. DSF sample sizes left to right: 1360, 2113, 1256, and 2217. Data are shown as mean ± SEM. Comparisons between vehicle- and 5-HT-treatments or silent and OA groups made using Mann-Whitney U tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **(D)** Representative recordings SFs and OA at -45 mV after treatment with vehicle or 5-HT. Insets: enlarged sections from each trace. AP, action potential; DRG, dorsal root ganglion; DSF, depolarizing spontaneous fluctuation; MP, membrane potential; NA, nonaccommodating; OA, ongoing activity; RMP, resting membrane potential; SA, spontaneous activity. **Contributions:** Elia R. Lopez designed/performed experiments; Max A. Odem designed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments.


A
RMP
-45 mV
***
SA or OA incidence (%)
100
80
60
40
20
0
0/16
0/21
3/16
17/20
Vehicle
5-HT
Vehicle
5-HT
B
-45 mV
OA
DSFs >5 mV
****
****
Median AP freqyency (Hz)
Median DSF frequency (Hz)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5-HT
Vehicle
5-HT
C
-45 mV
****
****
DSF amplitude (mV)
4
3
2
1
0
Vehicle
5-HT
Silent
OA
D
-45 mV, Vehicle
-45 mV, 5-HT (OA)
MP (mV)
60
40
20
0
-20
-40
-60
-80
5 mV
1 s
1 s

4.9.2. Serotonin enhances depolarizing spontaneous fluctuations in nonaccommodating neurons

Depolarizing SFs occurred randomly after either vehicle treatment or 5-HT treatment (**Figs. 17D, 18A1** and **18B1**). 5-HT increased the number of medium amplitude (3-5 mV) and large (>5 mV) DSFs during each recording (**Figs. 18A2** and **18B2**). The number of large DSFs paralleled the number of APs evoked during the same 30 second samples (**Fig. 18B2**). As predicted [56], 5-HT treatment also significantly (and substantially) lowered the voltage threshold for AP generation (**Table 7**). This likely contributed to the increased percentage of DSFs 3 to 5 mV and especially >5 mV that triggered APs (**Figs. 18A2** and **18B2**). In addition, 5-HT treatment significantly decreased the rheobase (consistent with an increase in the frequency of large DSFs) (**Table 7**). In contrast to the effect of SCI on AP latency at rheobase, 5-HT did not decrease AP latency. However, because of the low frequency and stochastic occurrence of APs (and underlying DSFs), demonstrating possible effects on AP latency is likely to require a much larger sample size.

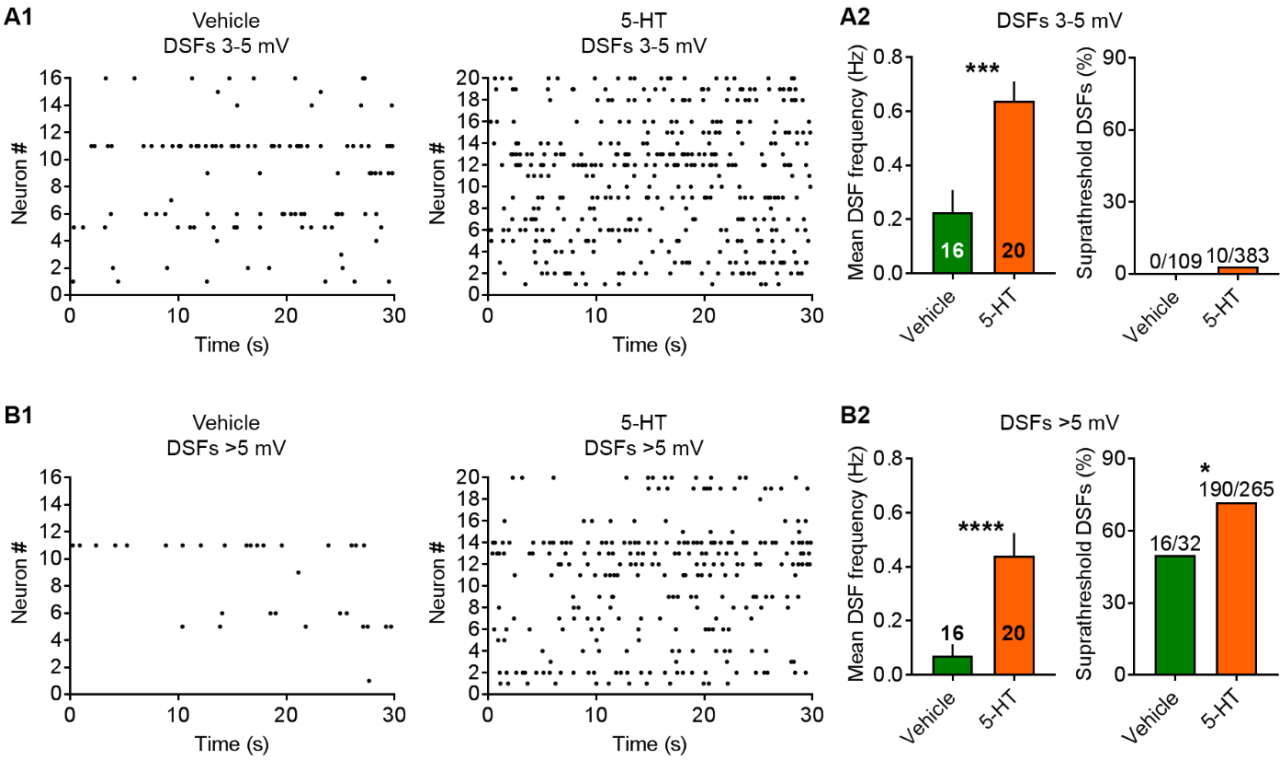


Figure 18. Serotonin increases the number of medium amplitude and large DSFs at -45 mV in NA neurons from naive rats. **(A1, B1)** Raster plots of medium amplitude and large DSFs during depolarization to -45 mV. Each row represents one neuron and each point a single DSF. **(A2, B2)** At -45 mV, 5-HT increased the number of medium amplitude and large DSFs, and the percentage of DSFs evoking APs. Bars represent the mean ± SEM or fraction in total sample, and significance was tested using Mann-Whitney U or Fisher exact tests. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. Neurons are from the naive rats (n = 4) used in **Figure 17**. AP, action potential; DSF, depolarizing spontaneous fluctuation; NA, nonaccommodating. **Contributions:** Elia R. Lopez designed/performed experiments; Max A. Odem designed experiments, analyzed data, and prepared figures; Edgar T. Walters designed experiments.

4.10. Conclusions and significance

The goal of these experiments was to define the electrophysiological specializations for low frequency, irregular OA in putative nociceptors associated with ongoing pain. A significant discovery in this study was the contribution of large DSFs (>5 mV, majority being suprathreshold) to persistent OA in NA neurons under SCI and inflammation-like conditions. The large DSFs are 1 of 3 functional aspects of membrane potential (others include depolarized RMP and hyperpolarized AP voltage threshold) that can promote an OA state in NA neurons. While this is not the first study to describe mechanisms driving OA in DRG sensory neurons (see oscillations in A-type neurons [7–9,223,224,384]), this is the first study to implement novel algorithms to quantitatively measure irregular SFs in membrane potential that drive OA in putative nociceptors. Furthermore, this is the first study to functionally segregate nociceptors based on specializations for generating OA and demonstrates that the OA state may be restricted to the NA type, not the RA type. The NA neurons dissociated from rats with a sham surgery exhibit modest signs of increased excitability. Given these alterations and the fact that artificial depolarization reveals a serotonin-induced potentiation of OA in NA neurons, one possible explanation for the observed sham-induced hyperalgesia in the MC test (*Chapter* 2) is that noxious probes elicit OA in probable C-fiber nociceptors; in short, severe peripheral damage to deep tissues may act as a priming event [183,300,301]. The noxious probes may cause sufficient depolarization in peripheral terminals of C-fibers to rapidly, and robustly, activate the nociceptors and generate a prolonged burst of APs (brief OA) that enhances evoked pain responses. Potentiation of OA in C-fiber peripheral terminals is one such mechanism by which serotonin and other inflammatory mediators may promote hyperalgesia. This raises an interesting possibility that serotonin and other inflammatory mediators may have stronger effects – and at lower concentrations – in NA neurons dissociated from rats with sham surgery or neural injury. Characterization of the neurophysiological basis of the stochastic AP discharge in the OA state under other pain-related conditions will potentially elucidate the

biophysical and signaling mechanisms governing the DSFs, thereby leading to the identification of new therapeutic targets for the treatment of many different forms of postsurgical and neuropathic pain.

Chapter 5: *Discussion*

5.1. Rethinking how to assess postsurgical pain in rodent injury models

Much of our understanding of the behavioral and physiological consequences of neural injury come from use of rodent models that involve substantial tissue damage in corresponding sham surgical procedures, yet little attention has been paid to postsurgical pain in these controls. Rather, separate rodent models have been developed for the explicit purpose of recapitulating surgical consequences of extensive tissue damage, and corresponding postsurgical pain studies exist tangential to many neuropathic studies. For example, behavioral hypersensitivity can be observed lasting a few days following incision of the skin and deep muscles in the plantar surface of the paw [43,386], about 2 weeks after laparotomy [136], up to 1 month after prolonged retraction of the skin and muscles in the thigh [122], >1-2 months following thoracotomy [49,173], and potentially longer following laminectomy [199,238] (see also *Chapter* 2). While some postsurgical models exhibit signs of acute [85,257,380] and subacute [173] ongoing pain, many postsurgical pain studies rely upon von Frey testing for assessing evoked mechanical hypersensitivity (for review [40]), and other types of behavioral tests are marginally used.

The MC test revealed the presence of postsurgical pain in sham-operated rats at 1-2 months and ≥3 months post-surgery for SCI, at <1 week post-surgery for SNT, and at 2 weeks post-surgery for CCI. The SCI experiments demonstrate that laminectomy can promote chronic postsurgical pain, long after the initial wound has healed. It is usual for rodent SCI studies to continue >1 month in order to adequately assess motor recovery using the BBB test and to perform von Frey reflex tests (for example see [24,57,58,90,171,196]). On the other hand, the SNT and CCI experiments demonstrate their respective sham surgeries acutely promote postsurgical pain at times (e.g., <1 month) when a majority of studies using PNI models typically perform von Frey reflex tests (for example [105,108,163,190,214,225]). Review of any

of the referenced studies will show little to no observed effects in sham control groups tested with von Frey filaments. It remains to be seen if any postsurgical pain due to sham surgeries for the SNT and CCI persist ≥1 month. Regardless, data suggest the MC test is sensitive for detecting postsurgical alterations in nociception and pain-avoidance behavior in 3 different rat models of surgically-induced neural injury, and may prove useful for studying transitional stages from acute to chronic pain. Besides the direct effects of the noxious probes, another possible explanation for the observed sham effects is that non-nociceptive stressors associated with conducting the MC test (e.g., handling, bright light) may have unmasked a postsurgical latent pain-like state that was potentially masked by μ-opioid-related mechanisms (see [52,221,281,305] and [346] for review). Additional studies are needed to adequately address this possibility. Indeed, current studies that report use of the MC test do not address its potential usefulness in assessing postsurgical pain or latent pain sensitization.

5.1.1. Factors that can occlude observation of postsurgical pain in rodents

Relatively mild rodent incision models and severe postsurgical injury models can show transient to chronic signs of increased behavioral hypersensitivity, ongoing pain, and nociceptor hyperexcitability [18,43,49,52,53,122,173,182,199,238,385,386]. Therefore, it is not unreasonable to expect that noxious mechanical stimuli, like the probes in the MCS, may reveal persistent postsurgical alterations in nociceptor sensitivity and evoked pain in sham-operated rodents. But why has a sham effect not been widely reported when noxious stimuli are used alongside or in place of innocuous stimuli? For one, the studies that use rodent injury models in the MC test do not include the proper sham controls [67,155,210,326]. Hogan has stated "…there are only modest effects evident in sham surgery control groups…", but at the time (2002) that statement was made it was primarily based upon knowledge gained from reflex tests of nociception [164]. There may be other confounding factors related to the use of these kinds of tests that have occluded observation of a sham effect. In studies using the von Frey filaments, rats that receive the sham surgery for a T13 hemisection SCI do not exhibit

mechanical hyperalgesia that is otherwise present in rats with SCI [147,149], but these studies make the highly unlikely assumption that ≤20 gram von Frey filaments constitute noxious stimuli. Naïve Sprague-Dawley rats have mechanical sensory thresholds upwards of 15-100 grams (see **Figures 5A** and **8C** in *Chapter* 2 and [90]), but many studies apply an arbitrary cutoff of 15 grams to replicate the "up-down" method described by Chaplan *et al*. [63]. It is not uncommon to encounter studies in which a ceiling effect is observed in the withdrawal threshold results (i.e., the naïve and sham groups are reported to have similar thresholds at ~15 grams; see also CCI experiments in *Chapter 2*). Other studies that use automated force delivery methods (e.g., Randall-Selitto analgesiometer) capable of administering noxious forces also suggest sham surgeries for clip-compression, transection, and contusive SCI do not induce mechanical hyperalgesia [89,272]. But again, like in the other studies using ≤20 gram von Frey filaments, Densmore *et al*. [89] assumed a maximum force of 20 grams is noxious and they did not use a naïve control group for comparison. Singh *et al*. [272] delivered maximum forces ≤1000 grams and describe their behavioral endpoint as when rats "vocalized or struggled vigorously". Thresholds for the sham group were lower compared to the naïve control group (~199 grams compared to ~210 grams) at 2 weeks post-surgery, but this effect was not statistically significant. It is unclear if sham-operated rats might have exhibited lower thresholds before the 2 week timepoint.

A seminal SNL study from Hogan *et al*. goes a step further and uses truly noxious stimuli (i.e., needle/pin-prick test) ([165] and see also [108,319]). Surprisingly, the Hogan *et al*. study reveals mild sham-induced hypersensitivity to innocuous touch and acetone, but the sham control group did not elicit hyperalgesic behavioral responses (e.g., sustained lifting, grooming) when probed with noxious stimuli. It is necessary to reiterate that in the needle/pin-prick test noxious stimuli are hand-delivered and the behavioral responses are not voluntary. In an effort to include a voluntary component in a follow-up study, Wu *et al*. designed a behavioral paradigm where rats could choose to avoid noxious stimulation by staying on a raised platform,

or jump down from the platform and receive a noxious stimulus to an injured hindpaw [375]. The rats with SNL learned to passively avoid the noxious stimuli, but sham-operated rats did not, presumably because they did not consider stimulation to be aversive. Again, noxious stimuli were hand-delivered and susceptible to unconscious bias [38]. More importantly, the authors only performed a skin incision for the sham surgery [375] (see also [238] for an incorrect sham control group in an unrelated study describing a postsurgical laminectomy model); the Wu *et al*. study is flawed because the sham surgery does not match the SNL surgery (i.e., the same deep tissues were not manipulated/damaged equally), and therefore the sham and SNL groups are not directly comparable nor is the "absence of effect" in the sham group truly conclusive. This oversight is not an isolated occurrence (see also [273]), and in some instances naïve or sham controls are outright omitted in studies using noxious stimuli (e.g., see [121,311,350]). It is possible that these confounding factors and those described in the prior paragraph have occluded observation of sham effects and postsurgical pain in studies using sham control groups for neural injury models.

Sham-induced postsurgical pain may also be a context-dependent phenomenon that is not revealed under conditions when noxious stimuli are hand-delivered. Stressful, investigator-induced analgesia and its effects on rodent withdrawal reflexes [334] is a recently described factor that may complicate interpretation of prior studies that use noxious stimuli as well as future studies if it is not properly controlled and reported. Unconscious bias [38] is another real possibility that can also influence hand-delivery of noxious stimuli similarly to von Frey filaments. Attempts to blind investigators can be complicated when rodents exhibit noticeable motor and/or postural deficits due to injury. Finally, standard reflex tests are not ethologically relevant as evoked withdrawals are not voluntary; the need for more tests that permit observations of voluntary behaviors has been expressed [344,392]. The MC test performed as a suitable workaround to these various issues: 1) both female and male experimenters conducted tests following appropriate handling and acclimation to the rats, 2) experimenters did

not need to hand-apply any noxious stimuli during tests, 3) scoring the videos – while blinded to the rats' surgery histories – for simple, objective behavioral metrics like number of crossings and latencies limited experimenter bias, and 4) the rats maintained a degree of autonomy within the confines of the MCS without any direct human interactions and/or influence.

5.1.2. Using the operant mechanical conflict test to assess postsurgical pain: Advantages, limitations, and future directions

The MC test may serve as an exceptionally sensitive test for evaluating signs of acute and chronic postsurgical pain in freely behaving rodents. Completion of this study has identified several areas of future research and applications as well as several limitations that will need to be addressed. Besides this study, rigorous testing with proper sham control groups is lacking but may prove useful for studying the underlying mechanisms associated with transitions from acute to chronic postsurgical pain (see [64]) in various neuropathic models. Analgesic efficacy of common treatments (e.g., gabapentinoids, opioids) for neuropathic pain may also need to be re-assessed in sham control groups of different types of injuries. Modifications to the MCS that outfit the probe chamber instead with dynamic hot or cold plates (see [21] for review) will allow for assessments of other modalities under more tightly controlled testing paradigms and contexts. The MCS is essentially a light/dark box that can also be used to assess comorbidities associated with pain (see [218,387] for review) like SCI-induced anxiety [14,232,236]. Preliminary experiments (unpublished observations) with SCI rats suggested pharmacological blockade of Nav1.8, and presumably nociceptor SA [174,389], had an anxiolytic effect when probes were absent; SCI rats spent more time outside of the dark chamber. One other surprising observation involved SCI rats with low to mid BBB scores (~10; occasional weight-supported stepping, no coordination) suddenly exhibiting carefully coordinated stepping behavior when introduced to the probes, suggesting that noxious stimulation can promote improved motor control after SCI.

Limitations of using the MCS most notably involve probe novelty, physical spacing, and learned aversion to the probes. Rats can quickly habituate to lower probe heights; some rats will exhibit notably high exploratory drives in the MCS (i.e., they cross frequently) while simultaneously developing novel crossing strategies to avoid placing their paws on the probes. Furthermore, the probes are simply not spaced close enough together to prohibit rats from learning to place their paws between them. I have observed many instances of rats (naïve, sham, and injured) placing their forepaws and/or hindpaws between the probes after enough exposure. Some of the sham and a majority of injured rats exhibited clear signs of a learned aversion to the probes. After the first exposure to 4 mm probes rats would refuse to cross the probes during a second trial, instead opting to face away from the probes against the far wall of the light chamber. Rats that exhibited this behavior would then explore the MCS without hesitation the following day when no probes were present, but reintroducing rats to the probes would prompt similar refusals to cross. These general observations suggest multiple tests with the same rats may not always be feasible if one were to test the effects of any experimental analgesics after pre-treatment trials with probes present. The order of probe exposure does not seem to affect escape latency in CCI rats well-habituated to the MCS [155], but the results described in *Chapter* 2 demonstrate that investigators may need to make predictions at very specific time points post-injury and carefully limit probe exposures. The robust aversion observed in all three neuropathic injury models likely involves complex supraspinal mechanisms (see [271]) that are still open to investigation at acute and chronic time points post-injury.

5.2. Sham surgical procedures, like severe peripheral injuries, are possibly priming-like events

The behavioral and electrophysiological results collectively feed into a larger narrative pertaining to nociceptor sensitization and priming. These topics have been extensively reviewed [183,301,358]. Nociceptive sensitization is an adaptive response to injury with

proposed survival benefits (for review [50,355,358]). This was not experimentally confirmed until recently; injury-induced nociceptive sensitization and SA in advanced cephalopods [6,82,83] promotes survival during predator attacks [81]. Many lower invertebrate phyla exhibit signs of nociceptive sensitization [357,359,372] as it was likely selected for early on during evolutionary development. Price and Dussor discuss the implications and potential difficulty in battling against the evolutionarily ancient machinery that underlies nociceptive sensitization in our efforts to curtail chronic pain [289]. Despite these difficulties, more recent work into mechanisms of priming has proved fruitful in identifying specific molecular signals that underlie nociceptive sensitization and transitional stages from acute to chronic pain. In the hyperalgesic-priming model posited by Reichling and Levine [301], injection of an inflammatory cytokine (e.g., prostaglandin E2; PGE_2) in the paw of a naïve rodent normally leads to a rapidly-resolving hyperalgesic behavioral response in the von Frey test. However, if a naïve rodent is exposed to an initial priming stimulus (e.g., carrageenan injection in the paw) ~1 week ahead of injection of PGE_2, then the behavioral response to PGE_2 will be more robust and long-lasting. This primed state depends upon protein kinase C epsilon (PKCε) activity; inflammatory and other stressful neuropathic insults trigger a switch from protein kinase A-mediated to PKCε-mediated signaling. Notably, Bavencoffe and Li *et al*. demonstrate that PKA-mediated signaling is still important for maintenance of persistent nociceptor hyperexcitability and SA following SCI [25]. These molecular switch-like mechanisms may be important in many pain conditions and disorders that involve some component of cellular stress. In light of this, the sham surgical procedures used as controls for many neural injuries can be considered a priming event. The damage to deep tissues and localized inflammation near peripheral nerve terminals may be tapping into some of the same molecular machinery, thereby adding an additional layer of complexity not previously – or at least fully – considered when using neural injury models. Indeed, the Levine and Price groups have speculated on the possibility that postsurgical pain is the result of a surgery-related priming event (i.e., tissue damage and/or localized inflammation) [183,300,301].

5.2.1. Sham procedures for surgically-induced neural injury models: Similarities, differences, and pain-related outcomes

The three neuropathic pain models used (SCI, SNT, and CCI) represent severe, clinically relevant injuries at distinctly different anatomical levels, each of which yield partial or complete axotomy of populations of nerve fibers. Yet, results in *Chapter* 2 suggest that the varying levels of injury severity did not necessarily add much in regard to effect size of behavioral measures in the MC test. The sham surgeries were sufficient to suppress exploratory behavior, but did not exhibit strong effects in the von Frey reflex tests with innocuous mechanical stimuli. What other insults (i.e., tissue damage) are commonly shared between the three sham surgeries that might account for the behavioral similarities between sham-operated and injured rats? If so, are they pertinent to clinical conditions of postsurgical pain conditions? The SCI and SNT require bluntly dissecting and retracting the paravertebral muscles to reach the thoracic and lumbar vertebra, respectively. The SCI and SNT also require significant damage of and removal of bone; the T10 dorsal process and lamina are removed during laminectomy and the L6 transverse process is removed to expose the L4/L5 ventral rami. Damage to the paravertebral musculature can promote degenerative lumbar kyphosis [68]; kyphotic deformities are relatively common adverse surgical outcomes [145,250] that can contribute to postsurgical pain in patients [363]. Spinal instability due to laminectomy can also contribute to signs of postsurgical pain in rodents [199,238], but the T10 lamina was not fully removed during laminectomy in my experiments and the degree of instability was likely miniscule. Meanwhile, the SNT and CCI require manipulation of the L4/L5 spinal nerves and sciatic nerve, respectively. Nerve manipulation (e.g., exposure, dissection) can cause moderate to severe discomfort as well as development of persistent postsurgical pain (see orofacial nerve manipulation [5,202]). The BBB score [24] effectively removes sham (T10 laminectomy) rats that unknowingly receive minor contusions during the laminectomy procedure, therefore the SCI sham surgery does not typically involve direct manipulation of the spinal cord itself if

performed correctly. The CCI surgery is notably less damaging than the SCI and SNT as it does not require any removal of bone, but it does involve blunt dissection and retraction of deep muscles in the thigh to expose the sciatic nerve. Besides the initial skin incision – that produces only transient effects on nociceptors [386] that cannot reasonably account for the SCI and CCI results observed at 1-3 months and 14 days, respectively – the only insults that are commonly shared between the three surgeries are the blunt dissections/retractions of deep muscles and inflammation due to surgery. Local tissue damage and inflammation certainly have powerful effects on nociceptors (see below and for review [23]). An underappreciated postsurgical pain model demonstrates that incision and retraction of the skin/muscles in the medial thigh is sufficient to cause persistent mechanical hypersensitivity without causing a dramatic increase in a neuronal injury marker in the DRGs [122]. The mechanical hypersensitivity is also reversible by administration of morphine and gabapentin [123]. These studies suggest that PNI models whose sham surgeries require unavoidable damage to musculature in the thigh *ought to regularly present* positive signs for a pain-like state (i.e., mechanical hypersensitivity) at 3 days to 3+ weeks post-surgery. Why this basic effect of skin/muscle incision and retraction on reflex sensitivity to innocuous mechanical stimuli is not always apparent – or at least described – in sham control groups reported in other pain-related studies may be partially due to inconsistencies in behavioral testing methodologies and reporting.

Unfortunately, reported experiments were not necessarily focused on the differences/similarities in the sham surgical procedures and consequences thereof. Molecular assays that quantify the degree of muscle deterioration (e.g., muscle weight, myofibrillar protein count; see [69]) and inflammation (e.g., B_1 and B_2 bradykinin receptors in muscle tissue; see [247]) were not performed, but it would be interesting to explore the relation between postsurgical musculoskeletal pain (for review see [40,246]) and inflammation with pain-

avoidance behavior in the MC test in future experiments. It is plausible that some of the postsurgical pain unveiled by the MC test is due to muscle strain and damage.

5.2.2. Neurophysiology of primed nociceptors

Whether or not the NA and RA neurons described *in vitro* can be reasonably called nociceptors must be addressed first before considering their functional relevance *in vivo* in the MC test and coming sections of the discussion. Capsaicin and IB4 identify TRPV1-expressing and non-peptidergic nociceptors [60,132,268,282–284,336,377], respectively, and were used to identify a subset of the NA and RA neurons. A majority of NA and RA neurons (70% and 67%, respectively) responded to capsaicin under voltage or current clamp with a capsaicin-evoked inward current or depolarization of membrane potential. Between 50 to 70% of the NA and RA neurons bound IB4, respectively. See also [377] and [28] for probable NA neurons responding to low concentrations of capsaicin and binding IB4, respectively, under similar conditions. The small soma diameters for the NA and RA neurons correspond to similar demarcations for nociceptors *in vitro* [132,282,283]. While the NA and RA monikers likely include some subpopulations of non-nociceptive DRG sensory neurons with similar discharge patterns, large majorities that show capsaicin sensitivity and bind IB4 strongly suggest both neuron types are comprised of putative nociceptors, and will be referred to as such through the remainder of the discussion.

Assuming the electrophysiological results collected from naïve, sham (T10 laminectomy), and SCI rats roughly parallel – the experimental manipulations (i.e., surgical procedures) were the same between studies – the behavioral results for the same groups in *Chapter* 2, then it is plausible to infer that postsurgical pain and related changes in avoidance behaviors in the MC test may have been mediated by changes *in vivo* in NA nociceptors. Several pieces of evidence collected *in vitro* coalesce to indirectly support this hypothesis: 1) sham surgery increased excitability in NA nociceptors, 2) SCI increased excitability in NA

nociceptors to an even greater degree in a graded fashion, and in additional metrics, 3) RA nociceptors isolated from sham and SCI rats exhibited little to no increases in excitability, and 4) “naïve” NA nociceptors pretreated with 5-HT exhibited increased excitability and generated more OA following extrinsic stimulation.

The amount of SA generated by NA nociceptors in the sham group was notably less prevalent than the SCI group (~19% vs ~59%, respectively), but the uptick in SA over the naïve group (~3%) and significant reduction in rheobase (see also [28]) indicated the sham surgery was sufficient to increase excitability in NA nociceptors. There were additional SCI-induced effects on NA nociceptors not caused by sham surgery (see **Table 5**). The fact that SCI selectively increased excitability in NA nociceptors supports the plausibility that the behavioral effects may be mediated by NA nociceptors; like in the behavioral tests, there was a graded response in electrophysiological measures and the sham group was often “between” the naïve and SCI groups. As an additional and novel measure of nociceptor excitability, the DSF analysis demonstrated that SCI-induced SA and depolarization-induced OA in NA nociceptors are both driven by large DSFs. Three functional aspects of membrane potential synergistically promote SA and OA in NA nociceptors (**Fig. 19**): 1) a prolonged depolarization of RMP, 2) a lowered AP voltage threshold, and 3) enhancements in the amplitude and/or frequency of DSFs. Looking at the sham group as a whole (see **Table 5**) suggests sham surgery did not robustly depolarize RMP or lower the AP voltage threshold. However, a sizable proportion ~15-20% of NA nociceptors isolated from sham-operated rats exhibit similar excitability profiles like that shown in **Table 6** (unpublished observations). These sham NA nociceptors that generate SA invariably exhibit alterations in one or more of the following: RMP, AP voltage threshold, and/or rheobase. Because the majority of NA nociceptors taken from sham rats do not exhibit these alterations, they drown out other NA nociceptors that do become more excitable. While the DSF analysis did not specifically focus on NA nociceptors isolated from sham-operated rats – rather, naïve and sham groups were combined to increase statistical power in comparison to

the SCI group – it is likely that DSFs are enhanced in amplitude and/or frequency in the select proportion of NA nociceptors that become more excitable due to sham surgery.

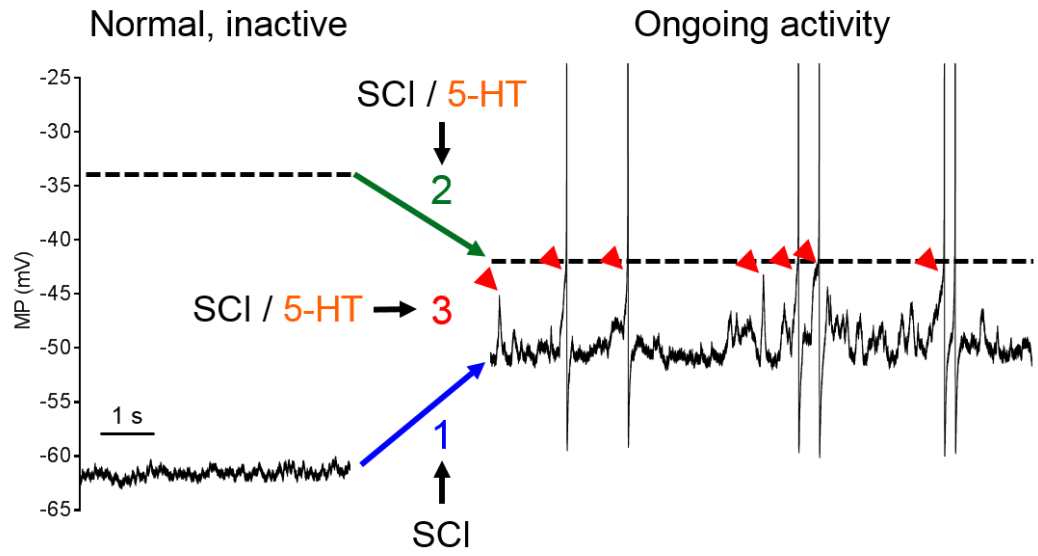


Figure 19. Summary of neurophysiological specializations that promote OA in NA nociceptors. The OA can be entirely intrinsic and thus completely spontaneous (denoted as SA) or extrinsically driven. Nociceptor OA *in vivo* may be driven by acute or ongoing exposure to extrinsic drivers of activity, sometimes combined with long-lasting intrinsic alterations. Representative recordings from two NA nociceptors illustrate the normal inactive state (sample from a naïve rat) and the OA state (sample of SA from a rat with SCI). Compared to the normal state, the OA state is marked by 3 alterations: 1) depolarized RMP (blue arrow), 2) decreased AP voltage threshold for (green arrow), and 3) increased amplitude and frequency of DSFs (red arrowheads indicate DSFs >5 mV, which are highly likely to elicit APs). Serotonin (5-HT, orange) potentiates OA by decreasing the AP voltage threshold and enhancing the DSFs. Both the inter-DSF intervals and interspike intervals between APs are irregular in the OA state and the discharge does not accommodate. 5-HT, serotonin; AP, action potential; DSF, depolarizing spontaneous fluctuation; MP, membrane potential; NA, nonaccommodating; OA, ongoing activity; RMP, resting membrane potential; SA, spontaneous activity; SCI, spinal cord injury. Figure prepared by Max A. Odem.

There was a modest trend for a SCI-induced decrease in rheobase in RA nociceptors, but the degree of change (~26% decrease in RA nociceptors, ~49% decrease in NA nociceptors) and post-SCI values (~125 pA vs 45 pA, respectively) were noticeably in favor of NA nociceptors being more excitable and susceptible to alterations by bodily injury. A thorough DSF analysis was not performed on the RA nociceptors as they were encountered far less often than NA nociceptors during experiments. However, the fluctuations appeared to be more regular in waveform during artificial depolarization (i.e., having oscillatory components, general observations), suggesting the fluctuations may be mechanistically distinct from those generated by NA nociceptors. An analysis of the DSFs in RA nociceptors will be informative for future mechanistic studies when the functional relevance of the RA type becomes more apparent. The RA nociceptors may overlap with and include the Aδ class of sensory neurons, as indicated by their slightly larger diameter and membrane capacitance. A-type sensory neurons generate rapid, oscillatory bursts in membrane potential that trigger bursts of APs [7–9,223,224,383]. However, none of the RA nociceptors generated SA or OA under any conditions tested (e.g., no fluctuations or oscillations generated bursts of APs at -45 mV) and their functional relevance is uncertain. No other trending SCI or 5-HT-induced effects on RA nociceptors were observed.

Due to the limited evidence of altered excitability and uncertain functional relevance of the RA nociceptors, it is *currently* more plausible to attribute behavioral changes *in vivo* to changes in NA nociceptors. This is further supported by experiments demonstrating NA nociceptors were sensitive to low concentrations of an inflammatory mediator, serotonin. Pretreating “naïve” DRG sensory neurons with 5-HT selectively increased excitability in NA nociceptors. The AP voltage threshold and rheobase were both significantly lowered, and the amount of OA generated in response to depolarization to -45 mV dramatically increased from 19% in the control condition to 85% in the presence of 5-HT. First of all, these results parallel other *in vitro* observations of 5-HT-induced excitability in peripheral fibers and DRG sensory

neurons [55,56,133,222,231,307,316,317]. Secondly, these results demonstrate the functional capabilities of the NA nociceptors *in vitro*, which parallel other *in vivo* and *ex vivo* observations of 5-HT-induced sensitization and activation of C-fibers, pain-related behavioral responses, and synergism with other inflammatory mediators (e.g., bradykinin, PGE_2) [2,62,161,166,222,252,275,307,313,341] (see also [230,332] for review). In light of these parallels, I predict the damage to deep tissues and inflammation associated with sham surgery and neural injury may act upon NA nociceptors in a similar manner and sensitize them to extrinsic stimuli, like the noxious probes in the MC test.

5.3. Functional significance of the NA nociceptors: Evoked pain, ongoing pain, or both?

I have addressed how postsurgical pain in rodents has been somewhat overlooked and how the avoidance behaviors elicited in the MC test relate to postsurgical pain. I have also touched on the plausibility that changes in NA nociceptors mediate behavioral changes *in vivo*, possibly in the MC test. Still, I have not established a clear functional role for NA nociceptors *in vivo* under postsurgical or neuropathic pain conditions, the types of sensory modalities and pain they may mediate, and pathways in which they may operate. Since nociceptor OA is a likely driver of central sensitization, then circuits in the dorsal horn are critical for appropriately modulating input from nociceptor OA in a functionally relevant manner for somatosensory processing. Indeed, where different types of nociceptors project in the dorsal horn tells a great deal about their sensory modalities and functional significance. I will attempt to establish a functional role and propose a theoretical model of NA nociceptor activity by addressing the following questions:

1. Is NA nociceptor activity generated *in vitro* relevant *in vivo*?
2. Is the MC test assessing an evoked or ongoing pain-related behavior?
3. Is that behavior mediated by different subpopulations of NA nociceptors and driven by OA?

5.3.1. Activity generated by NA nociceptors *in vitro* is relevant *in vivo*

Patients often describe ongoing/spontaneous pain as being the most discomforting and debilitating, yet some of that pain may be unknowingly evoked by the patient during their daily routines. The mechanisms underlying evoked and spontaneous pain in many different neuropathic and inflammatory pain conditions are unclear, and definitions of what constitutes truly spontaneous pain is still open to debate [30]. The strongest links between conscious reports of pain by patients – some of which have peripheral neuropathies – and activity in C-fibers are from microneurography studies [195,262,263,269,270,323,324]. Activation of C-fibers is reported to evoke burning and/or aching pain sensations, and spontaneous activity in C-fibers is considered an optimal readout for neuropathic pain [312,314]. For technical review see [128,321]. Microneurography recordings also demonstrate that multiple rat neuropathic models (three PNI models, two polyneuropathy models in this particular study) have incidences of spontaneously active C-fibers that are similar to those of patients with peripheral neuropathies [322]. Many rodent neuropathic and inflammatory models have spontaneously active C-fibers [3,28,105,249,330,374,379,386], and the SA correlates with pain-related behaviors like spontaneous foot lifting [105,386]. Direct activation of C-fibers, ablation of C-fiber populations, or knockdown of Nav1.8 aptly demonstrate the role C-fibers play in pain-like states in animal models [26,86,362,389]. In light of these details, it is plausible to infer that the spontaneously active C-fibers described *in vivo* encompass some proportion of the NA nociceptors that show a functional capacity for OA *in vitro*.

The specializations that promote the low frequency, irregular OA and SA in NA nociceptors were elucidated purely *in vitro* and found to be intrinsic to isolated DRG neuron somata (see also [25,28,377,389]). There are notable concerns about the artificiality of this model system, but isolated DRG neuron somata retain their properties observed *in vivo* [17,132,154]. The contusive SCI model was specifically chosen due to previous experiments and experimental advantages that make the study of nociceptor OA more feasible

[25,28,57,377,389]: 1) most importantly, SCI-induced nociceptor OA has been described as being generated in/near the DRGs *in vivo* and in an excised nerve preparation, 2) a majority of nociceptors at and below the level of the injury enter into an hyperexcitable state and generate SA, 3) the hyperexcitable state is observable from 3 days to ~6-8 months post-SCI, and 4) the model yields a large quantity of DRG tissue that is otherwise not available when using other injury models – like SNT or CCI – in which only 3 of the DRGs innervate the injured hindlimb. Although the SNT and CCI models were not the focus of my electrophysiological experiments, it is very likely that specializations (e.g., large DSFs) for NA nociceptor OA are present in many other ongoing pain conditions (unpublished observations of large DSFs in nociceptors taken from rats with SNL or chemotherapy-induced peripheral neuropathy, mice with SCI, and humans with cancer-related chemotherapy pain). These specializations appear to manifest *in vitro* in multiple conditions, across multiple species (see also nociceptor SA in squid following peripheral injury [82]), specifically in putative nociceptors isolated under neuropathic ongoing pain conditions. Furthermore, irregular OA with similar firing frequencies is generated by C-fibers *in vivo* following SCI [28]. These repeated occurrences suggest the specializations are likely to be relevant *in vivo* rather than purely coincidental, represent generalized mechanisms that may have evolved specifically to promote low-level peripheral input to the CNS, and may facilitate stimulus-evoked ongoing pain as well as truly spontaneous pain.

5.3.2. Ongoing pain may not influence avoidance of noxious stimuli in the operant mechanical conflict test

The type of pain being assessed by the sharp probes in the MC test is probably evoked by activation of nociceptors that respond to mechanical stimuli, but to what degree does ongoing, truly spontaneous pain modulate behavior during the test? Can evoked and ongoing pain be distinguished in this test? The recent push for more ethologically relevant, unbiased operant measures for pain has begun to reveal the importance of distinguishing spontaneous from evoked pain, as spontaneous pain is a more informative translational tool for assessing

preclinical neuropathic pain over standard reflex tests [256,286]. One clear, unbiased distinction between sham and SCI rats is that SCI promotes the development of chronic, spontaneous pain as measured by the operant CPP test [389] (see also *Chapter* 3). Axotomy of the lower lumbar spinal nerve(s) – using the original Kim and Chung procedure [163] or modified procedure [101] – also results in spontaneous pain that is detectable in the CPP test [19,159,192,291]. The equivalent sham-operated rodents do not exhibit spontaneous pain as revealed by the CPP test. Notably, deep plantar incision is sufficient to also produce spontaneous pain transiently [85] when incision-induced hypersensitivity and nociceptor SA are likely to be present [386]. Despite these distinctions in regards to spontaneous pain, both the sham and injured rats tested in *Chapter* 2 developed a robust aversion to the noxious probes likely due to nociceptive sensitization and enhanced evoked pain.

Although spontaneous pain was not explicitly assessed in *Chapter* 2, it is likely that at 3 days post-surgery the sham and SNT rats were experiencing acute, ongoing postsurgical pain [85,386]. Unexpectedly, SNT rats exhibited a clear reduction in crossings compared to naïve and sham rats when no probes were present. Some SNT rats exhibited hyperalgesic-like responses [165] and were observed to guard their injured, allodynic hindpaw when moving about the MCS. They also likely had altered gait patterns (see SNI example [326]). This may be due to SA in the intact L4 DRG C-fibers that drive pain-related behaviors like spontaneous foot lifting [101,386]. Djouhri *et al*. did not use sham controls and but did posit that C-fiber SA is less likely to be present in shams (see [235]), but the study by Xu and Brennan did use the appropriate sham control (i.e., skin incision alone without cutting deeper fascia and muscle) for their incisional pain model. Although mechanical allodynia is driven by mechanisms of central sensitization, including SA originating from axotomized A-fibers in the L5 DRG [224,225], the spontaneous pain-related behavior is more likely to be driven by intact C-fiber SA induced by Wallerian degeneration of nearby myelinated fibers [101,374]. It cannot be ruled out that stepping with the allodynic paw elicits a spontaneous-like evoked response, which may be

similar in nature to the “spontaneous” pain evoked by daily routine (see [30]). Regardless, the only observations that distinguished the effects of neural injury from sham surgery were of SNT rats crossing fewer times without probes. All other notable differences were in sham-operated and rats with neural injury compared to naïve, uninjured controls in the presence of noxious probes. This suggests that any neuropathic-induced ongoing/spontaneous pain, while likely present and contributing to the rats’ pain state, may not be a strong determining factor of pain-avoidance behavior.

5.3.3. Possible sensory modalities and pathways for NA nociceptors

Unfortunately, none of the experiments described directly link the NA nociceptors to any particular sensory modality – besides maybe temperature due to the high incidence of capsaicin-sensitivity – or with their prospective projection sites in the dorsal horn. However, a few reasonable interpretations about potential projections and function can be gleaned from the limited marker information. To refresh, a majority of the NA nociceptors responded to capsaicin (~70%) and roughly half (~49%) bound IB4, indicating NA nociceptors are primarily comprised of $TRPV1^{+}$ subclasses of nociceptors. There was insufficient data to comment on overlap between capsaicin sensitivity and IB4 binding in the NA nociceptors. However, Usoskin *et al*. posit an unbiased classification scheme for primary sensory neurons in mice using single-cell RNA sequencing [349]. Species differences aside, they show TRPV1 expression overlaps with three distinct populations of unmyelinated DRG sensory neurons, two nonpeptidergic and one peptidergic. Gene ontology maps suggest these three populations mediate itch, inflammatory-related itch, mechanical, heat, neuropeptide function, and pain sensory properties. Here, Usoskin *et al*. define pain as a sensory property using the following descriptions (see supplementary data for [349]): 1) sensory perception of pain, 2) response to pain, 3) detection of temperature or chemical stimulus involved in sensory perception of pain, and 4) behavioral response to pain. As for the NA nociceptors that did not respond to capsaicin, Usokin *et al*. also show there are two distinct populations of unmyelinated $TRPV1^{-}$ DRG sensory neurons, the C-

type low-threshold mechanoreceptors (LTMRs) that uniquely express tyrosine hydroxylase (see also [219]) and neurons that jointly express the Mas-related G protein-coupled receptor member D (gene name *Mrgprd*) and purinergic P2X ligand-gated ion channel 3 receptor (P2X3, encoded by the *P2rx3* gene). These distinct populations are largely consistent with other reported populations of nociceptors and their projections in the dorsal horn (for review [279,284]). In light of this information, it is probable that a majority of NA nociceptors that are TRPV1$^+$/peptidergic or TRPV1$^+$/non-peptidergic project in the superficial layers, laminae I and outer laminae II, and mediate a collection of itch, mechanical, heat, and inflammatory sensations. Any NA nociceptors that are C-LTMRs will project into the deeper layer of laminae II, and likely not innervate glabrous skin (see [219]). Finally, any NA nociceptors that are TRPV1$^-$/*Mrgprd*$^+$ will project deep within laminae II and mediate mechanical sensations.

5.3.4. A proposed model for pain-avoidance behavior and postsurgical pain mediated by NA/*Mrgprd*$^+$ nociceptors

Our current understanding of the *Mrgprd*$^+$ nociceptors may offer deeper insight into NA nociceptor function and pain-avoidance behavior in the MC test. The *Mrgprd*$^+$ nociceptors selectively innervate the skin epidermis, mediate mechanical pain and itch, and are topographically organized in deeper regions of laminae II in the dorsal horn [26,61,111,176,360,399,406]. The *Mrgprd*$^+$ nociceptors express the P2X3 receptor [406] and are excited by adenosine triphosphate (ATP) *in vitro* [111]. Activation of keratinocytes stimulates ATP release [274,404], suggesting keratinocytes are intermediaries for activation of *Mrgprd*$^+$ nociceptors and mechanical pain (see also [187,342] for review). Ablation of *Mrgprd*$^+$ nociceptors fails to eliminate “spontaneous” pain-related behaviors caused by formalin [328]. This is consistent with other studies showing ablation of *Mrgprd*$^+$ nociceptors leads to specific deficits in sensation of mechanical stimuli [61,399]. When optogenetically activated, the *Mrgprd*$^+$ nociceptors that innervate the plantar surface of the paw mediate withdrawal [266]. Note, the *Mrgprd*$^+$ nociceptors in the glabrous skin are innately more sensitive than the *Mrgprd*$^+$

nociceptors innervating hairy skin in the upper thigh, suggesting some *Mrgprd*$^+$ nociceptors are somatotopically organized for responding to mechanical stimuli encountered during normal ambulation. A sizable proportion of *Mrgprd*$^+$ nociceptors also generate SA *in vivo* following chronic compression injury to the DRG [364] and exhibit enhanced excitability (e.g., depolarized RMP, lowered rheobase, more APs when stimulated) in a model of inflammatory pain and itch [292]. Deletion of *Mrgprd* decreases nociceptor sensitivity to cold, heat, and mechanical stimuli *ex vivo* and increases rheobase *in vitro* in cultured DRG sensory neurons [298]. Rau *et al*. also demonstrate application of an Mrgprd receptor ligand, β-alanine, lowers AP threshold and increases firing in *Mrgprd*$^+$ nociceptors, and posit β-alanine production in the skin can tonically activate *Mrgprd*+ nociceptors. These studies suggest *Mrgprd* may be important for enhancing nociceptor excitability in pain conditions involving epidermal tissue damage and peripheral inflammation. The *Mrgprd*$^+$ nociceptors exhibit injury-induced electrophysiological properties similar to some properties observed in the NA nociceptors, and the potentiated activity in response to β-alanine is reminiscent of NA nociceptor potentiated OA in the presence of 5-HT. Dussor *et al*. did not measure OA in dissociated *Mrgprd*$^+$ nociceptors treated with ATP [111], so it would be interesting to determine whether ATP potentiates OA like β-alanine or like 5-HT in NA nociceptors. Based on referenced studies (mostly in mice) and *currently* available electrophysiological data for NA nociceptors, I think it is reasonable to predict *Mrgprd*$^+$ nociceptors are activated by the sharp probes in the MC test, and that NA/*Mrgprd*$^+$ nociceptors mediate pain-avoidance behavior via mechanisms that promote OA (e.g., direct mechanical activation, secreted factors from keratinocytes, other inflammatory mediators). While TRPV1$^+$ nociceptors mediate some forms of mechanosensation in rats (e.g., pressure; see [44]), they do not appear to mediate pin-prick in naïve rats [44] or movement-induced pain in rats with bone cancer [158]. Peripheral terminals of *Mrgprd*$^+$ nociceptors innervate more superficial epidermal layers than TRPV1$^+$ terminals in mice [61,176,304,406]; TRPV1 is also seen near the dermal-epidermal junction in humans [335] and rats [146]. The precise relationships between MCS probe height, rat weight, and degree of skin displacement

are still unclear. Activation of TRPV1$^+$ nociceptors may not directly mediate probe-induced mechanical transduction, but that does not preclude their contribution should sufficient radial pressure activate them. However, based on my observations of forepaw and hindpaw placement during crossings it is unlikely rats apply sufficient body weight and pressure on the probes. Forepaw withdrawals during initial investigations are exceptionally fast and rats learn to raise the heels of their hindpaws and step between probes during crossings.

Higher-order processing in the pre-frontal cortex and hippocampus have been implicated as neurobiological correlates for pain-avoidance behavior in the MC test [271]. Whether or not the mechanosensory information is perceived as painful to naïve rats is still unclear; naïve rats exhibit longer escape latencies [155,271] but will repeatedly cross over low and high probes when given the opportunity, suggesting the experience is not so aversive to abolish exploration like in the sham-operated and rats with neural injury. In light of this, evoked discharges from NA/*Mrgprd*$^+$ nociceptors that propagate supraspinally may not take precedence during the decision-making process (**Fig. 20**). Probe avoidance probably becomes more advantageous – and exploration decreases – once a rat has undergone a serious peripheral injury involving damage to deep tissues (e.g., sham surgery) (**Fig. 21**). The proliferation and circulation of inflammatory mediators and other extracellular signals following injury can increase excitability in peripheral nerve terminals and somata of nociceptors [64]. In sham-operated rats, NA/*Mrgprd*$^+$ nociceptors may be sensitized and respond to the probes with a burst of OA (higher frequency, prolonged duration). Indeed, NA nociceptors isolated from sham-operated rats exhibit signs of increased excitability (e.g., reduced rheobase) and more SA. Whether or not OA in NA/*Mrgprd*$^+$ nociceptors is the sole driving force of the rats' behavior is unclear; the broader ensemble of TRPV1$^+$ NA/*Mrgprd*$^-$ nociceptors likely encodes a larger variety of sensory information. They may generate more intrinsic SA and inflammatory mediator-activated OA both in the peripheral terminals and somata. This may further enhance NA/*Mrgprd*$^+$ nociceptor activity; signal transmission may be augmented within the DRG and

nociceptor somata. Tissue injury and peripheral inflammation can stimulate infiltration of non-neuronal immune cell types in/near the DRGs (e.g., macrophages [356,358]) and activation of satellite glial cells – coupled interactions with satellite glial cells may enhance nociceptor excitability (see [34,35,109,168,191,212,388]). Following a more severe neural injury (e.g., SCI), the same pain-avoidance behavior is elicited, but the underlying mechanisms are possibly more complex (**Fig. 22**). For example, SCI promotes a hyperexcitable state and OA in nociceptor somata [25,28,377,389] and axons [57]. The capsaicin-sensitive, peptidergic nociceptors – probable NA/*Mrgprd*$^{-}$ nociceptors – are more likely to generate SA in their somata [28]. They might also generate more extrinsically driven OA. Low frequency SA and extrinsically-driven OA generated in NA/*Mrgprd*$^{-}$ nociceptors and other mechanically insensitive nociceptors [195,263,288,323] are probably important for maintenance of central sensitization in dorsal horn circuits [369] and strengthening of synaptic connections. Indeed, peptidergic DRG sensory neurons show signs of injury-induced growth states [27,229]. Increased sprouting and proliferation of post-synaptic elements may allow these nociceptors to infiltrate into deeper laminae and increase circuit excitability (see [1,72,200,216,267,365] for sprouting of peptidergic primary afferents). This model(s) of NA nociceptor function and pain-avoidance behavior in the MC test is merely speculation at this point. It will be interesting to see how future studies map NA nociceptors onto current classifications based on molecular markers, function, and anatomy in the spinal cord. Future studies that differentially manipulate populations of TRPV1^{+} and *Mrgprd*$^{+}$ nociceptors in rodent models of neural injury in combination with using the MC test to assess pain-avoidance behavior will further elucidate the functional roles of these nociceptors, and possibly identify new therapeutic targets specific to postsurgical and neuropathic pain.

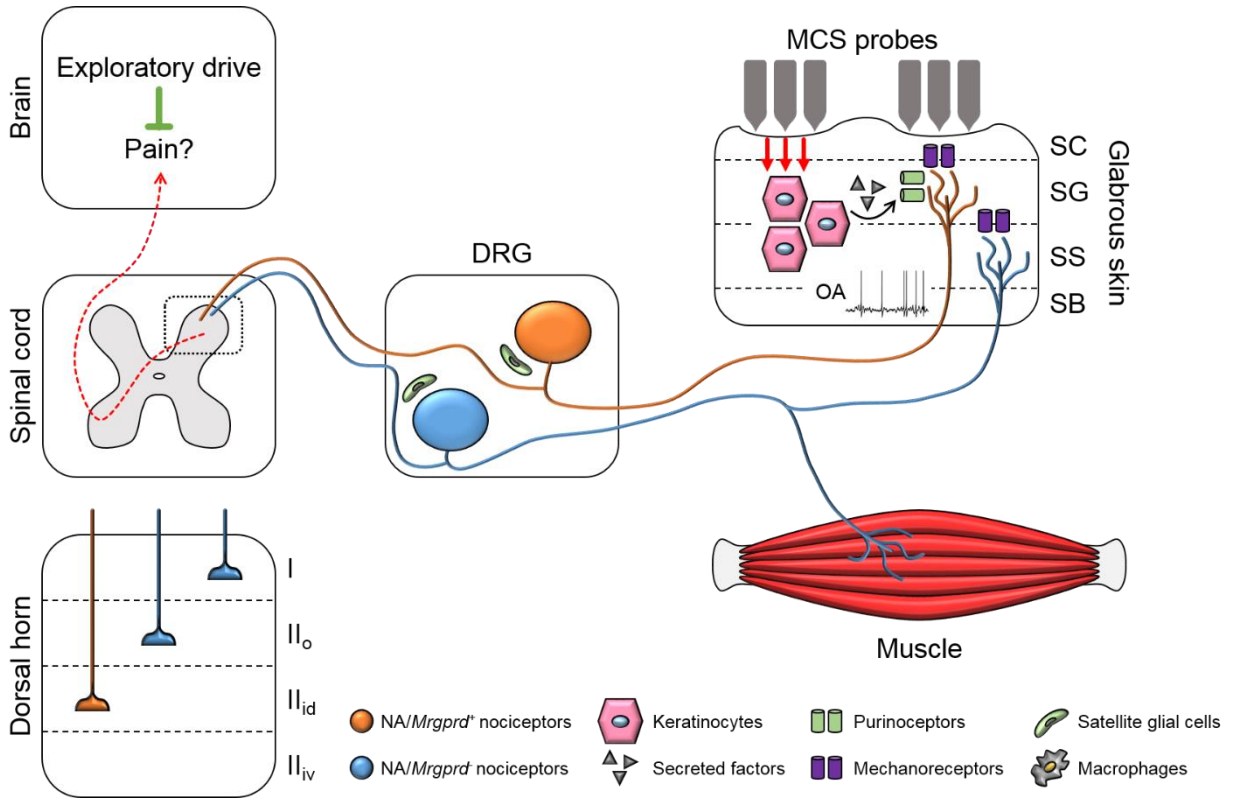


Figure 20. Proposed model for NA/*Mrgprd*$^+$ nociceptor function under naïve conditions. I-II$_{iv}$, dorsal horn laminae; DRG, dorsal root ganglia; MCS, mechanical conflict system; *Mrgprd*, Mas-related G protein-couple receptor D; NA, nonaccommodating; OA, ongoing activity; SB, stratum basale; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum. Figure prepared by Max A. Odem.

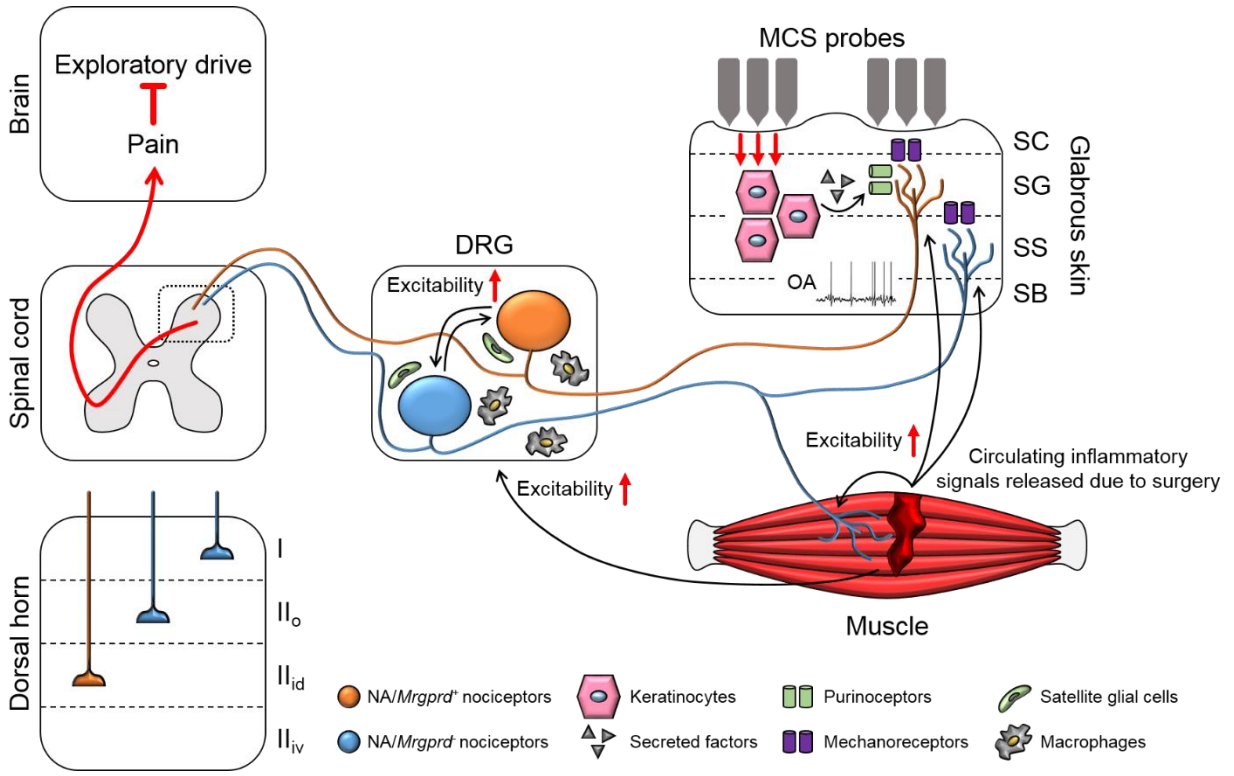


Figure 21. Proposed model for NA/*Mrgprd*$^+$ nociceptor function following peripheral injury conditions (e.g., sham surgery). I-II$_{iv}$, dorsal horn laminae; DRG, dorsal root ganglia; MCS, mechanical conflict system; *Mrgprd*, Mas-related G protein-couple receptor D; NA, nonaccommodating; OA, ongoing activity; SB, stratum basale; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum. Figure prepared by Max A. Odem.

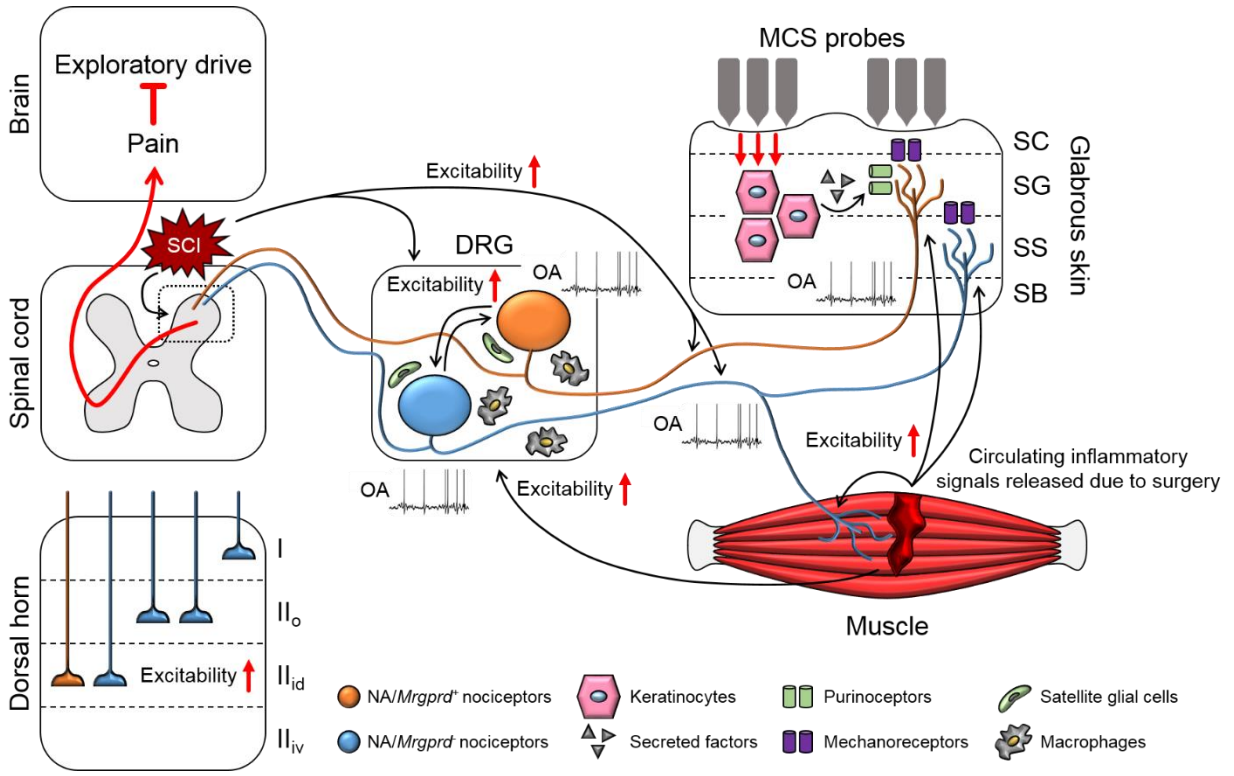


Figure 22. Proposed model for NA/*Mrgprd*$^+$ nociceptor function under severe neural injury conditions (e.g., SCI). I-II$_{iv}$, dorsal horn laminae; DRG, dorsal root ganglia; MCS, mechanical conflict system; *Mrgprd*, Mas-related G protein-couple receptor D; NA, nonaccommodating; OA, ongoing activity; SB, stratum basale; SC, stratum corneum; SCI, spinal cord injury; SG, stratum granulosum; SS, stratum spinosum. Figure prepared by Max A. Odem.

5.4. Concluding remarks and future directions

Geoffrey Bove bluntly repudiates the von Frey test as he questions its validity as an accurate measure for pain, naming it the “tin standard” in the pain field [38]. In summary, he firmly emphasizes the threshold measurements described in many studies are likely not comparable due to differences in experimental methods and human bias. By the end of my time as a graduate student, I also question the validity of the von Frey and other subjective reflex tests for similar reasons. My experiments demonstrate the von Frey test does not reveal acute or persistent forms of postsurgical pain in three different neuropathic injury models. This is consistent with a large literature base, and not in a good way. In 2004, Mogil and Crager suspected the pain field’s exclusive dependence upon reflex tests would be untenable [255]. If the results from the MC test are to be believed, then there is a real possibility that prior interpretations of some experiments using rodent neural injury models – which were dependent upon reflex tests – may be obscured if postsurgical pain in sham control groups was not properly taken into account. This includes interpretations of underlying pain-related mechanisms and treatment-related effects. Indeed, there are issues concerning the reproducibility and replicability of research [29,115,180,290] as well as translational efficacy in the pain field [392].

Increasing emphasis on more descriptive, automated behavioral tests (e.g., MC and CPP tests) and recording techniques that provide more natural approximations of nociceptor activity *in vivo* (e.g., dorsal root and nerve recordings; see [28,135,348]) might lessen “death valley’s” gap. Future studies that link nociceptor activity *in vivo* with the negative affective-motivational components of pain might provide more suitable backdrops for testing experimental therapeutics involving cellular and molecular mechanisms predicted to drive nociceptor activity and pain behavior.

Appendix

Conditioned place preference methods

The three compartment conditioned place preference (CPP) device (Med Associates, Inc., Fairfax, VT, USA) is comprised of a neutral center grey chamber and two larger, solid colored chambers – one white, one black – for contextual pairing of treatments. Manual guillotine doors separate each chamber. The white and black chambers were dimly illuminated while the grey chamber was brightly lit to discourage rats from preferring it over the other two chambers during baseline and post-conditioning tests. The manufacturer provided MED-PC v4.34 software automatically tracks rat location and time in each chamber via infrared photo beams. Data from two simultaneously running CPP devices were collected and stored on a Windows 7 Dell desktop computer.

Test procedures were followed as described in [19,192]. Briefly, rats were permitted full access to all 3 chambers for 15 minutes to assess innate preference. Rats that spent >80% or <20% of their time in any of the 3 chambers were excluded from experiments due to innate chamber bias. The next morning, rats were injected with saline vehicle (i.p., 2 mL/kg volume) and after 10 minutes placed in the innately preferred chamber for 30 minutes (black chamber for majority of rats). That afternoon (~4 hours later) rats were injected with gabapentin (100 mg/kg, i.p., 2 mL/kg volume dissolved in 0.9% saline; Toronto Research Chemicals, Ontario, Canada) and after 10 minutes were placed in the less-preferred chamber for 30 minutes. The next day, rats were permitted full access to all 3 chambers for 15 minutes to assess changes in chamber preference. No treatments were given at this time. The CPP score (in seconds) is the time spent in a chamber in the post-test minus the time spent in the same chamber in the pre-test.

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Vita

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