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Behavioral measures of persistent pain in mice

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Abstract

The use of animal models in the understanding of the neurobiology of pain perception is essential for the development of new pharmacotherapies. Yet, in modeling human clinical experience, there is a lack of meaningful and appropriate dependent variables of pain-related behaviors in the mouse. In this thesis, we address the problem of finding behavioral measures of pain in mice that fully encompass the range of experience inherent to human pain conditions. Therefore, in this work complex behaviors were assessed as potential measures of persistent pain in mice

We investigated a wide range of quality of life behaviors in three classic pain models: spared nerve injury, chronic constriction injury and injection of complete Freund's adjuvant. Mechanical hypersensitivity is prominent in each of these conditions and persists for many weeks. To assess more complex behavioral outcomes, home cage behavior was continuously monitored after injury and a battery of motor disability and affective behavior tests were performed on these mice. No model of chronic pain produced long-lasting changes to behaviors of daily life, either in the home cage or in tests of affect and disability.

Next, we observed behaviors in three other models of persistent pain: osteoarthritis, disc-degeneration, and dental pulp exposure. In a pilot study of the former two models, mice with joint degeneration were tested for locomotor ability and motivation, but showed no signs of disability. Lastly, we measured behavior in the setting of dental pulp inflammation, for which there is no standard method of measuring pain levels. As with other models, pulpal injury also did not impact behavior in the home cage. Instead, we used an operant assay of sucrose consumption as a measure of dental pain in mice. Data from this task suggest that pain can, in fact, influence some elements of complex behavior. However, as alteration in daily life activities is the feature that is so disrupted in patients with chronic pain, our results suggest that many murine pain models do not fully reflect the human conditions and raise questions regarding the limitations of these models in pain research.

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Chapter 1

Understanding human pain using mouse models

Everyone experiences pain. Though unpleasant, acute pain can be protective — an alert to a potential threat or to prevent further injury. In the clinic, acute pain is readily treated with the available drugs, such as opioids or acetaminophen. Conversely, chronic pain often has seemingly no adaptive benefit and is more problematic to treat. Though of no evolutionary utility, the incidence of chronic pain conditions is quite high in the human species, ranging from 12 to 30% of the population in the United States and Europe (Breivik et al., 2006; Johannes et al., 2010). Given this high prevalence and difficulty in the management of chronic pain, understanding its underlying mechanisms using animal models is essential for discovering better therapeutic targets.

A mechanistic understanding of pain

Any discussion of chronic pain must be informed by an understanding of the circuits that underlie the experience. Though seemingly different, chronic and acute pain (which refers to both momentary sensations as well as longer, but temporary, manageable sensations such as post-operative pain) experiences derive from the same neural circuits. In both cases, as a sensory and emotional experience, the pain sensation begins with detection in the peripheral sensory afferents and continues through to brain. Nociception, that is the activation of peripheral nociceptors

(sensory afferent neurons that detect noxious stimuli) and their communication with the central nervous system (Loeser et al., 2008), has been well studied in rodent models of pain. Once nociceptive afferents send pain information into the dorsal horn of the spinal cord, the pain signal is then relayed via projection neurons to many areas of the brain, including thalamic nuclei, parabrachial and amygdala (Braz et al., 2005). The brain regions involved in pain processing are representative of the diverse experience of pain from sensory discrimination in the somatosensory cortex to the emotional involvement of the limbic system. As the focus of this dissertation is the complex behavioral outcomes of the pain experience, theses regions will be discussed in more depth below.

The boundaries between acute, persistent and chronic pain are not distinct (Woolf et al., 1998), though clearly the time scales can be very different. A more precise way to distinguish between different pain conditions is by the specific changes that occur in the affected tissue, the nervous system or a combination of both. Using this approach, there a four main categories of pain sensation: nociceptive, inflammatory, neuropathic, and the combination of inflammatory and neuropathic, sometimes called dysfunctional (Costigan et al., 2009). Nociceptive pain primarily refers to acute, noxious sensory detection, wherein the high-threshold primary afferent is directly activated by external (thermal, chemical, or mechanical) or internal (such as ischemia) stimuli. Nociceptive pain can be an important element of some chronic pain conditions — osteoarthritis, for example (Costigan et al., 2009).

In chronic pain, often times tissue damage causes the release of inflammatory mediators, which in turn leads to the sensitization of the peripheral nociceptors. This enhanced afferent input, in addition to a direct action of the cytokines released from the site of inflammation, can increase the excitability of neurons in the spinal cord, known as central sensitization. As a result, stimuli that were once innocuous now cause pain (Huang et al., 2006; Hucho et al., 2007; Woolf et al., 2000). However, in this case, once the tissue has healed and the immune response returns to normal, the pain generally stops. The exceptions to this are chronic inflammatory diseases, such as rheumatoid arthritis, where inflammation persists without specific tissue injury.

Neuropathic pain, defined as pain after a lesion or disease of the peripheral or central nervous system (Baron, 2006), also involves increased sensitivity and activity of the primary afferent. Additionally, long-term changes in neuronal gene transcription, changes in excitatory/inhibitory synapses in the spinal cord, and neuroimmune response all lead to the induction and maintenance of neuropathic pain conditions (Baron, 2009; Hansson, 2006; Scholz et al., 2007; Woolf et al., 2000). Lastly, there are chronic pain conditions that are complex combinations of these mechanisms such as cancer (Mantyh, 2006), osteoarthritis (Kidd, 2006; Wieland et al., 2005), fibromyalgia (Julien et al., 2005; Staud et al., 2006), and back pain (Deyo et al., 2001).

A clinical understanding of pain

In considering the study of pain using animal models, it is vital to ground those models in an understanding of the epidemiology and pain outcomes in the human population. Indeed the impetus for animal pain research is the major impact chronic pain has as a major health problem worldwide; not only harming those suffering from it, but also costing billions of dollars in missed work and health care (Holden et al., 2003; Stewart et al., 2003). Reports estimate about a third of the population in the US suffers from some occurrence of chronic pain, with studies ranging from 15 to over 50% (Johannes et al., 2010). In a large study of European countries, the percent of a country's population reporting chronic pain ranges from 12-13% in Spain, Ireland and the UK to much higher incidences of 25-30% in Italy, Poland and Norway (Breivik et al., 2006). Given the array of different chronic pain mechanisms and conditions, studies of the incidence of specific types and locations of pain are of interest. Figure 1 shows the distribution of causes and locations from one large study (Breivik et al., 2006). Also of interest to the study of pain is that often the incidence of specific chronic pain conditions varies with age (Figure 1) and differs between men and women.

The assessment of human pain for both research and treatment necessitates the use of tools that reliably measure both pain sensation and affect. But pain is a subjective experience, so researchers must rely on self-report, though other methods, such as brain activation (imaging and EEG) may also be used. There are a wide range of questionnaires and other scales used to quantify the otherwise subjective pain

experience. The most basic assessment simply asks the patient to rate their overall pain on a numeric scale from "no pain" (0) to "the worst pain imaginable" (10) (Huskisson, 1974; Scott et al., 1979). Some questionnaires focus on the quality of the pain, asking the subject to rate the words that describe the pain such as "sharp", "gnawing", or "tiring" to allow for the categorization of pain symptoms (Dworkin et al., 2009). Still other pain inventories, in addition to questions of pain rating, also ask for the extent to which the pain interferes with different aspects of life — such as mood, walking ability, normal work, social relationships, sleep, and enjoyment of life (Daut et al., 1983; Keller et al., 2004). These assessment tools are constantly being translated and validated across many populations(Bouhassira et al., 2009).

Pain assessment that includes impacts on daily life suggests another key feature of clinical chronic pain: its close association with impairments to affect, cognition, and functional disability. A majority of chronic pain patients report an impact on at least one measure of daily life (figure 1) and often are unable to perform at normal work levels (Breivik et al., 2006; O'Connor, 2009; Stewart et al., 2003). Experimental studies have also shown that in human chronic pain can negatively alter performance in cognitive, attentional, and emotion-related tasks (Apkarian et al., 2004a; Solberg Nes et al., 2009). Similarly, in clinical surveys of pain patients, the presence of chronic pain is associated with lower quality of life scores (Allen, 2003; Bailey et al., 2010; Lamé et al., 2005; Liedberg et al., 2009; Pilowsky et al., 1985) and found to often be co-morbid with affective disorders such as depression and anxiety (Bailey et al., 2010; Elliott et al., 2003; Geisser et al., 2000; Gormsen et

al., 2010; Price, 2000). Interestingly, there are a few competing theories regarding to the relationship between chronic pain and depression. One suggestion is that it is a direct causal link: the pain causes depression, either through the involvement of the hypothalamic-pituitary-adrenal (HPA) axis (Blackburn-Munro et al., 2001) or, in the case of inflammatory pain, through the actions of cytokines in the CNS (Raison et al., 2006). However, more recently, there is evidence that the level of disability may be the most important predictor in the development of depression with chronic pain (Miller et al., 2010; Stegmann et al., 2010).

Given the close association then between chronic pain and affective disorders, it is therefore unsurprising that there is a great amount of overlap between brain regions involved in pain and emotions(Borsook et al., 2007). In fact, pain itself can be considered an emotion (Treede, 2006). Functional imaging data from humans suffering from chronic back pain show that activity in the rostral anterior cingulate, amygdala, posterior thalamus, and medial prefrontal cortex fluctuates with spontaneous pain rating of the subject during the course of the experiment (Baliki et al., 2006). Human studies also reveal that the ACC activity is related to the affective, unpleasantness rating of pain rather than sensory intensity ratings (Apkarian et al., 2005; Rainville, 2002). Pain not only impacts brain activity, but may, in fact, also alter its structure (Apkarian et al., 2004b; May, 2008).

Modeling chronic pain in rodents

What is an "animal model"?

In modern science, animal models are invaluable to the study of nearly all areas of the life sciences. For neuroscience in particular, work in rodents has lead to many links between genes, molecules, and behavior. But what does it mean to make an animal model? The term can be used to refer to simply the strain of mouse, particularly when using transgenic mutant strains of mice. Alternately, it can refer to the manipulation of the animal, such as surgery or injection. Lastly, it can refer to the tests and measures used to quantify behavior (Mogil, 2009). In pre-clinical pain studies, all these levels are relevant. However, for the sake of clarity in this work, I will refer to the middle level, that is the manipulation, as a "model;" and the last level, as "measures", "assays", "tests," or "outcomes". Though the primary, "subject" level is clearly essential to discuss, in the case of the work presented here, it will not be considered as "a model" (Table 1).

Studying pain in the totality of its definition as both sensory and affective experience generally requires the use of awake, behaving animals to truly measure any pain phenotypes. Other outcome measures such as anatomical changes and neuronal activity, using electrophysiology or c-Fos expression, still must be validated as truly relating to a behavioral phenotype. A measurable increase in fos expression in the anterior cingulate of a mouse, for example, means little regarding "pain" if you cannot also show that preventing the increase relates to the pain experienced by the animal. Therefore, it is essential to develop measures that

reflect pain in all its aspects and, without the ability to self-report, researchers must rely on behavioral "surrogates" of pain.

Rodent pain models

Just as human pain experience spans from beneficial, acute alert signals to maladaptive chronic pain, so too does the range of animal models of pain begin at acute nociceptive stimulation models to long-term degenerative models. While we focus here on chronic pain, it is important to understand the acute models, as they have often become a pain *measure* for the persistent models. These acute models most often involve applying a noxious stimulus, such as radiant heat or mechanical pressure, to an easily accessed body part, like the hindpaw and tail, and measuring withdrawal or other nocifensive behaviors. These are most often reflexes or innate responses and do not persist beyond the initial stimulus. Alternately, models that focus on persistent or chronic pain, by necessity of the condition they replicate, must endure for longer periods, though how long ranges from less than an hour to months (which is still of questionable validity to human chronic pain).

Animal models of inflammation, much as in human inflammatory conditions, are persistent, but not as long in duration as other etiologies of pain. These models most often involve the injection of substances that activate an immune response or, in the case of one model, inducing inflammation with UV-B irradiation (Bishop et al., 2007). One of the most commonly used models, the formalin test, has been well used because of the prominent outward signs of discomfort the animal shows

(licking and flicking of the injected limb) and its remarkably reproducible biphasic response (Tjølsen et al., 1992).

More prolonged inflammatory models are often meant to reproduce arthritis-like conditions. In rats, immune activating substances, such as carrageenan or complete Freund's adjuvant are injected directly into a joint (most often knee, ankle, or tempromandibular) to produce a monoarthritic condition (Butler et al., 1992; Harper et al., 2000; Neugebauer et al., 2003). In mice, where the joint space is harder to accurately target, intraplantar injections into the hindpaw are often used to model inflammatory pain (Honore et al., 2000). In humans, purely inflammatory arthritis, i.e. rheumatoid arthritis, is not generally limited to one joint. Thus, systemic injections of adjuvant have been given to induce polyarthritis (Cain et al., 1997; Calvino et al., 1987), but concurrent overall ill health of the animals can confound the behavior of these animals.

Pain caused by neuropathy, either injury- or disease-related, is a difficult condition to treat. Therefore, in an effort to understand the mechanisms, many pre-clinical models of neuropathic pain have arisen. By definition, neuropathic conditions all involve some damage to the nervous system (typically peripheral injury) whether caused by mechanical injury, disease, or drugs. While we focus here on injury-induced neuropathies, there are well-characterized models of diabetic-, HIV- and chemotherapeutic- induced painful neuropathies (D'Almeida et al., 1999; Tredici et al., 1998; Tsuda et al., 2008) (Bölcskei et al., 2005; Tredici et al., 1998; Wallace et al.,

2007). In these and the injury-induced models, the assessment of pain has, until recently, focused on changes in sensitivity.

Considering the relative rarity of injury-induced neuropathic pain in humans, there is an astonishing array of methods to produce neuropathy, though all but a few involve the sciatic nerve. The remaining models are most often lesions to the nerves of the orofacial region (Khan et al., 2010). The most common of these involve either nerve ligations or a constriction (Benbouzid et al., 2008; Shields et al., 2003). Surprisingly, some of the most used methods involve somewhat inexact methods, such as ligating "1/3 to 1/2" of the sciatic nerve (Seltzer et al., 1990) or tying a "loose" ligature around it (Bennett et al., 1988). This may lead to variation in the extent of injury to each animal and might account for different outcomes across research groups. Interestingly, though these models all have similar lesions to the same nerve, when they are directly compared, different injuries often yield different levels of behavioral and anatomic outcomes (Dowdall et al., 2005; Roeska et al., 2008). Because of this, researchers often use more than one method of nerve injury.

Other types of pain conditions, though no less prevalent in humans, have fewer animal models, particularly those in specialized tissue. These include models of osteoarthritis (OA), headache, back, dental, and cancer pains. Models of joint degeneration in the knee (OA) and back (disc-related disease) have been induced using mechanical disruption of the joint or chemical induction of degeneration (Ameye et al., 2006; Singh et al., 2005). Mechanical damage is also used to induce

dental pain in rodents, sensory information from which is predominately nociceptive, given the almost exclusive innervation by small diameter neurons (Khan et al., 2010; Mason et al., 1985). To induce cancer-related pain models, researchers often implant cancer cells into the bone (the most common and severe location of human cancer pain)(Schwei et al., 1999). Lastly, two common human pain conditions — headache and fibromyalgia — currently have no reliably validated (at least behaviorally) rodent model.

Measurement of pain in animals

While the choice of chronic pain model is clearly important to preclinical studies, just as essential are the behavioral measures used to assess the pain experienced by the animal. Finding adequate measures of pain in animals is vital not only to pre-clinical research but also to veterinary medicine where the lack of obvious pain behavior can lead to untreated distress or the overmedication where no pain is present (Roughan et al., 2003). To date, the main behavioral outcomes of pain in preclinical research are evoked measures of hypersensitivity, the limitations of which are addressed below and are a key motivator of this thesis. Additionally, there are three other categories of assessment methods: operant, spontaneous, and complex behavior; which are potentially better predictors of the complete pain experience, not just nociception.

Evoked behavioral outcomes of pain in rodents include withdrawal reflexes or simple reflexive behavioral responses, such as jumping, scratching, licking, and

biting in response to a specific stimulus — such as radiant heat or pinch. Recently, the lack of clinical relevance of these measures to assess "chronic pain" in rodents has been raised (Blackburn-Munro, 2004; Mogil et al., 2004). Though chronic pain in humans is often accompanied by a change in mechanical or thermal sensitivity, much more problematic is spontaneous and use-based pain (Backonja et al., 2004). Therefore, the reliance on hypersensitivity/evoked behavior, while more simple to perform and straightforward to analyze, may be an incomplete measure of pain. This is particularly because, as a reflexive measure, it ignores the affective component of pain. The only evoked measures that are suggestive of this component are vocalizations in response to noxious stimuli (Williams et al., 2008), or innocuous stimuli in the setting of injury (Han et al., 2005).

While evoked measures in large part ignore the affective component of pain, operant models have arisen specifically to address the integration of sensory and motivational components. However, most of these tests still involve a specific stimulus to motivate behavior (Baliki et al., 2005; Mauderli et al., 2000; van der Kam et al., 2008). Operant tasks are comprised of a learned response stemming from a positive or negative reinforcer. In particular, conditioned place aversion (CPA) and preference (CPP) have been used in the setting of chronic pain models to show that after an animal learns to associate one environment with a painful stimulus they will then, when given a choice, spend less time in that environment (or more, if the stimulus was analgesic) (Johansen et al., 2001; Sufka, 1994). A variant of CPA has been used in the setting of neuropathic pain, with one area associated with

stimulation of the ipsilateral hindpaw, which the rat then avoids in favor of the area with contralateral stimulation (Deyama et al., 2007; Pedersen et al., 2006). Other operant methods have included self-administration of analgesics (Colpaert et al., 2001; Richardson et al., 1996). However, operant models are limited by the ability of the animal to learn the task, which is particularly problematic for mouse studies. Results of these studies are also often confounded by motivation and the affective state of the animals.

Because of the importance of spontaneous, on-going pain sensations in clinical chronic pain, researchers have tried to measure the potential spontaneous (and use-based) pain. To this end, direct observations of an animal after induction of a pain model have been used to account for behaviors that might be suggestive of spontaneous pain. This includes not just simple behaviors that are observed by eye, but also recording vocalizations (Jourdan et al., 2002; Ko et al., 2005) and simple disability tests such as weight bearing (Bove et al., 2003). Among the many specific behavioral outcomes recorded after different pain types, the most common are flinching, licking, guarding, grooming, scratching, autotomy, and postural changes (Calvino et al., 1987; D'Almeida et al., 1999; Dowdall et al., 2005; Kupers et al., 1992; Minert et al., 2007). But the use of gross recordings of behavior has proven difficult, particularly in the case of monitoring models of neuropathic and inflammatory pain. In a recent paper from Mogil, et al., a review of the literature reporting spontaneous pain changes reveals that animals often spend a remarkably small percent of time (<10%) in the measured behavior compared to the overall observation time (Mogil

et al., 2010). Work from the same group observed that pain in mice can be measured using a facial grimacing scale with some models of persistent pain, such as formalin and the acetic acid visceral pain test. However, they were unable to use grimacing as an outcome of spontaneous pain in two neuropathic pain models (Langford et al., 2010). This suggests that spontaneous measures may be limited in their use in preclinical models of chronic pain.

Lastly, the use of complex behaviors to assess pain in animals has recently been a larger area of focus in pre-clinical studies. This effort derives from the notion that if the animal models of chronic pain are truly modeling a chronic pain experience, they will have changes to daily life similar to those in humans. Therefore, these include quality of life behaviors like those discussed previously: daily life activities; as well as conditions often co-morbid with chronic pain such as affective, cognitive, and social changes (Table 3). Indeed, in the rat, researchers have observed changes in anxiety, depression, attention, cognition, functional disability, exploration, sleep, and social interaction (Gonçalves et al., 2008; Hasnie et al., 2007; Kontinen et al., 2003; Kontinen et al., 1999; Leite-Almeida et al., 2009; Monassi et al., 2003; Roeska et al., 2008; Schütz et al., 2009).

While many of these complex behaviors have been successfully explored in the rat, there is far less evidence that pain can be measured using these tools in the mouse. Here, we have used measures of pain beyond simply evoked responses in a number of different chronic pain modalities. In Chapter 2, we assess the utility of a wide

range of quality of life behaviors for their potential as measures of pain in three of the most standard mouse models of chronic pain. Chapters 3 and 4 concern models of pain that do not have standard hypersensitivity measures. We investigate the use of measures of disability in back and knee pain and daily life behaviors in animals with dental injury.

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Figure 1

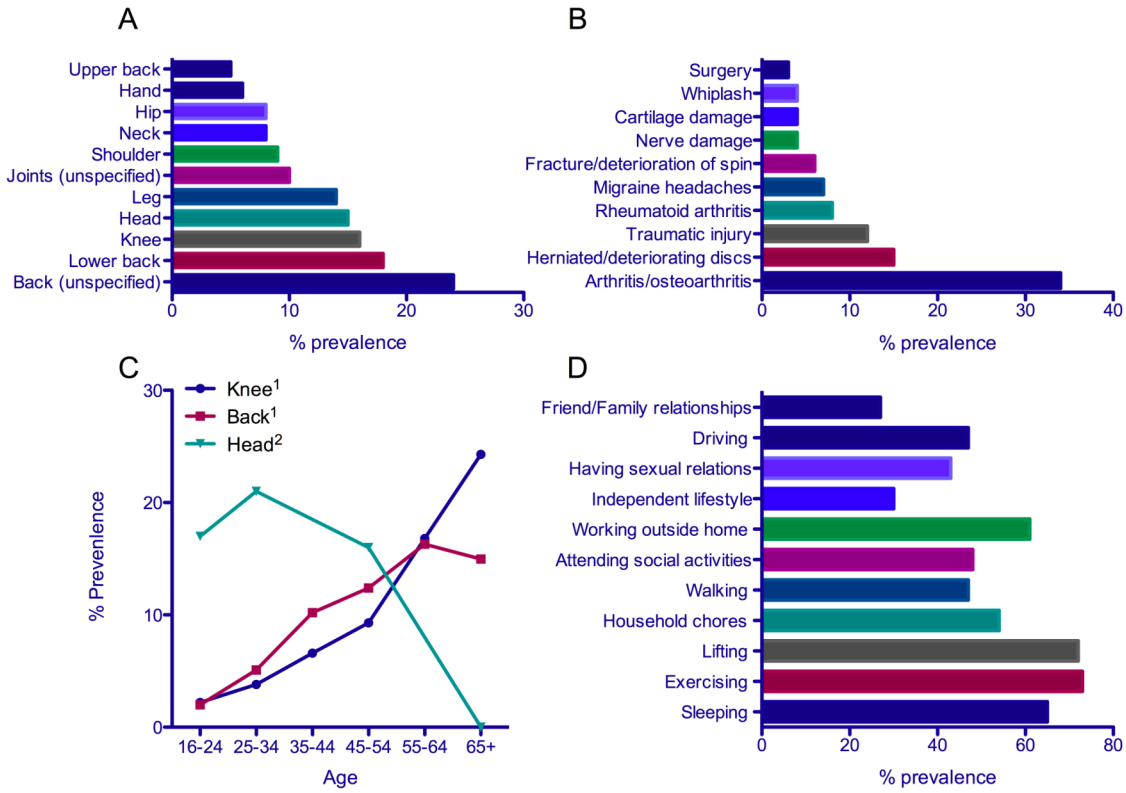

Figure 1. (A-B) Percent prevalence of those reporting pain in a large European survey, by location of pain (A) and cause (B), figure adapted from Breivik, 2006. (C) Different types of chronic pain are age dependent, adapted from two sources [1]Badley, 1992 and [2]Von Korff, 1988. (D) Percent prevalence of those with chronic pain who responded "less able" or "no longer able" to performing different daily life activities from the same European study as in A and B. (Badley et al., 1992; Breivik et al., 2006; Von Korff et al., 1988)

Table 1. Different definitions of an animal "model". Adapted from Mogil, 2009.

Subject	Model	Assay/ Measure
Species	Manipulation (cutaneous application, surgical procedure, injection)	Reflex/Evoked
Strain		Spontaneous
Mutation	Location/Body Part	Operant
Sex	"Chronic" or Acute	Complex behavioral outcomes (e.g. sleep, cognition, disability)
Age		

Table 2. Quality of life measures used in humans and possible ways of testing the measure in mice.

Dimensions of quality of life behaviors	
Human	Mouse
General activity	locomotor behavior in the home cage
Mood	Tests of affective state (anxiety, depression)
Walking ability	dynamic weight bearing, gait analysis
Normal work (outside the home and housework)	home cage activity, nest building, digging
Relations with other people	Social interaction, aggression, mating
Sleep	EEG
Enjoyment of life	anhedonia
Eating and enjoyment of food (orofacial)	food consumption

Chapter 2

Behavioral indices of ongoing pain are largely unchanged in male mice with tissue or nerve injury-induced mechanical hypersensitivity

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Abstract

Despite the impact of chronic pain on the quality of life in patients, including changes to affective state and daily life activities, rodent preclinical models rarely address this aspect of chronic pain. To better understand the behavioral consequences of the tissue and nerve injuries typically used to model neuropathic and inflammatory pain in mice, we measured home cage and affective state behaviors in animals with spared nerve injury (SNI), chronic constriction injury (CCI) or intraplantar CFA. Mechanical hypersensitivity is prominent in each of these conditions and persists for many weeks. Home cage behavior was continuously monitored for 16 days in a system that measures locomotion, feeding and drinking

and allows for precise analysis of circadian patterns. When monitored after injury, animals with SNI and CFA behaved no differently from controls in any aspect of daily life. Animals with CCI were initially less active, but the difference between CCI and controls disappeared by 2 weeks after injury. Further, in all pain models, there was no change in any measure of affective state. We conclude that in these standard models of persistent pain, despite the development of prolonged hypersensitivity, the mice do not have significantly altered "quality of life". As alteration in daily life activities is the feature that is so disrupted in patients with chronic pain, our results suggest that the models used here do not fully reflect the human conditions and point to a need for development of a murine chronic pain model in which lifestyle changes are manifest.

Introduction

The perception of pain is not simply a sensory, nociceptive experience, but one that often disrupts a patient's quality of life (O'Connor, 2009). Despite this, animal studies have focused on measures of hypersensitivity in the setting of tissue or nerve injury (Mogil et al., 2004). Unquestionably, mechanical and thermal hypersensitivity are often associated with human chronic pain disorders (Baron et al., 2009; Gottrup et al., 1998; Maier et al., 2010), but often more disruptive to patients is spontaneous, ongoing pain (Backonja et al., 2004). The latter is difficult to document in animals, and thus rarely studied. Chronic pain disorders are also associated with changes in affective state, such as depression and anxiety (Bailey et

al., 2010; Geisser et al., 2000; Price, 2000), and overall quality of life measures (Bailey et al., 2010; Liedberg et al., 2009; Pilowsky et al., 1985); again few of these conditions are studied in preclinical models.

Spontaneous pain is particularly difficult to measure in rodents. Animals occasionally display measurable signs of pain, such as flinching or guarding in some pain models, but this has proved hard to detect in persistent pain models (D'Almeida et al., 1999; Jourdan et al., 2002; Kupers et al., 1992; Langford et al., 2010; Mogil et al., 2010; Wallace et al., 2005). Further, if the animal is indeed experiencing spontaneous pain similar to humans, there should be an impact on its daily life activity and affective state, behaviors we will herein refer to as "quality of life" measures. To date, such studies in rodents have concentrated on the rat and included measures of daily life such as sleep (Guevara-López et al., 2009; Kontinen et al., 2003; Landis et al., 1988; Monassi et al., 2003; Schütz et al., 2003; Tokunaga et al., 2007) and locomotion (Cain et al., 1997; Hasnie et al., 2007a; LaBuda et al., 2001; Matson et al., 2007; Vonsy et al., 2009). Results from these and other studies on affective state changes following chronic injury are mixed. For example, some studies only reported changes in depression-like behavior (Gonçalves et al., 2008; Hu et al., 2009), others only found alterations of anxiety-like behavior (Hasnie et al., 2007a; Ji et al., 2007; Roeska et al., 2008; Seminowicz et al., 2009), and still others found changes to both (Leite-Almeida et al., 2009) or none (Kontinen et al., 1999). There are even less data for the mouse, despite its utility in genetic studies. As in the rat, there are conflicting reports as to whether nerve injury-related pain leads to

an increase in anxiety in mice (Hasnie et al., 2007b; Matsuzawa-Yanagida et al., 2008; Narita et al., 2006a; Narita et al., 2006b; Suzuki et al., 2007) and only a handful of studies have focused on changes to daily life behavior with mixed results. A gastritis model revealed decreased locomotion, eating and drinking (Painsipp et al., 2007). A sciatic nerve cuff model of neuropathic pain found no change in daily life activities, but did report alterations in measures of affect (Benbouzid et al., 2008).

The paucity of studies monitoring the daily life of animals in pain undoubtedly reflects the complexity of long-term monitoring of behavior. Video recording of mice in their home cage has been used (Houghton et al., 1997), but the time required to analyze the data limits this method. In the present study, we used a new approach that allows for automatic and detailed analysis of home cage activity of mice over an extended period (Goulding et al., 2008). Our objective was to determine the extent to which tests of daily activity and affective state can be used as surrogate measures of pain in mice using current standard models of persistent pain, spared nerve injury (SNI), chronic constriction injury (CCI) and intraplantar Complete Freund's Adjuvant (CFA). Because of the known variability among mouse strains in their expression of pain behavior, we studied two inbred strains (Balb/c and C57Bl/6). We monitored daily activity in the home cage and also measured performance in a variety of behavioral tests of affective state, for up to 5 weeks following injury.

Methods

Animals

Adult male mice (Balb/c and C57Bl/6) were purchased from Jackson and Charles River laboratories, and arrived at least 2 weeks before testing began. All mice were housed in cages with corncob bedding and a cotton nestlet in groups of 3 to 5, unless separated due to fighting issues, which occurred occasionally in both strains. All cages were changed every two weeks, at least two days before the next behavioral test. Animals had freely available food and water under a standard 12-hour light/dark cycle with a regulated ambient temperature of 20-22°C. Experimental manipulation occurred at 7-10 weeks of age. All procedures were approved by the Institutional Animal Care and Use Committee at UCSF and the guidelines of the Committee for Research and Ethical Issues of IASP. For all experiments, animals were habituated to handling prior to testing.

Experimental models of pain

Surgeries were performed under isoflurane anesthesia. Spared nerve injury (SNI) was performed as described previously (Shields et al., 2003) with two of the three branches (sural and common peroneal) of the sciatic nerve ligated and cut. Sham controls received the same surgical procedure, except that the sciatic nerve remained undisturbed. Chronic constriction injury (CCI) was performed using a modification of the Bennett and Xie (Bennett et al., 1988) procedure. Briefly, the sciatic nerve was exposed at mid-thigh, proximal to its trifurcation. Two ligatures of

6-0 chromic gut suture were tied loosely around the sciatic nerve, 1-2mm apart. As in the SNI sham procedure, the sciatic nerve was exposed but not disturbed in the sham group and, for all groups, overlying muscle was closed with 6-0 silk suture and the skin closed separately with wound clips. Naïve groups were exposed to isoflurane anesthesia for a similar length of time as sham, SNI, and CCI animals. After surgeries animals were returned to their home cages in a mixed environment (generally at least two different groups per cage.)

For the model of inflammatory persistent pain, animals received an intraplantar injection of 10μL Complete Freund's Adjuvant (CFA) into the left hindpaw. To extend the length of time of the inflammation, a second 10μL intraplantar injection was given one week after the first. Controls received intraplantar injections of saline each time. For shorter duration persistent pain, animals received 5% formalin (in 20μL) intraplantarly. Sickness was induced by an injection of lipopolysaccharide (20μg in 200μL, i.p.). As the injuries are comparable to those that evoke inflammatory and neuropathic pain in humans, we refer to these conditions as models of inflammatory or neuropathic pain.

Mechanical hypersensitivity

For both home cage monitoring and affective behavior experiments, the mice were habituated to the testing chambers 2 to 3 times before baseline testing began. Testing chambers consisted of clear plexi-glass cylinders on a raised wire mesh grid. On each day of testing, the mice were first habituated to the testing cylinders 60-90

minutes before. We used von Frey filaments with the up-down method (Chaplan et al., 1994) to obtain the baseline mechanical threshold. For home cage monitoring groups, post-injury thresholds were obtained immediately before and after the monitoring period. Other animals were tested in weeks 1, 2 (3 for CFA experiments), and 5.

Home cage monitoring

To monitor behavior in the home cage, we used the automated monitoring system developed by Goulding, et al (Goulding et al., 2008) . This system consists of 32 cages (with betachip bedding, plastic niche and cotton nestlet) each placed on a pivoting platform with two load beams calibrated to detect position of the mouse, with photobeams at the feeder and a lickometer at the water bottle to detect bouts of feeding and drinking. While this experiment required the mice to be individually housed in the system, they were group housed until placed into the system. For each round of monitoring, the 32 animals were evenly divided between experimental and control (naïve and sham or, for the CFA experiment, saline-injected) groups. Experiments were done in Balb/c mice with CCI (n=20-22, in two separate runs), SNI (n=10-11) or CFA (n=16) and SNI in C57Bl/6 mice (n=10-11). For nerve injury experiments, animals were placed in the system at 48 hours after injury, to allow time for mechanical threshold testing. In the CFA run, monitoring began 48 hours after the second injection of CFA. In all experiments, monitoring proceeded continuously (except for 1.5-2 hours daily maintenance on the system) for 16 days. Using methods developed for the system, data were checked for errors

and activity classified as inactive or active. Within the active state, mouse behavior was further classified based on location, movement, and feeder/lick spout data as feeding, drinking, moving, or other (which includes small movements and can be separated by location.)

Short-duration behavioral tests of locomotor and affective state behavior

Groups of mice were run in a battery of activity and affective tests (not all tests were performed in every group, supplemental figure 5). Each test was run with an interval of at least 3 days, which has been shown to be sufficient so that behavior on one test does not influence the next (McIlwain et al., 2001; Paylor et al., 2006). For all short-duration behavioral tests, animals were brought into the testing room at least half an hour prior to beginning the test. Between tests, the testing apparatuses were sprayed with dilute bleach and wiped dry.

Tests of anxiety-like behavior

For all tests of anxiety-like behavior, animals were only studied once on each apparatus. The open field (OF) test of activity and anxiety was performed under normal lab lighting (more than 100 lux). Mice were placed in the OF apparatus, which consists of four white chambers measuring 50 × 50 × 38 cm, allowing 4 animals to be tested concurrently. Mice were allowed to freely explore the chamber for 30 minutes. Each chamber was divided into the outer zone (15 cm from the walls) and the center zone. Activity was recorded by video and analyzed using the Ethovision software. Time spent in the center zone was used as the measure of

anxiety. The elevated zero maze (EZM) is a modified elevated plus maze test of anxiety-like behavior that eliminates the ambiguous center square(Milner et al., 2008) . This maze consists of an elevated (42 cm), round platform (5.5-cm width) divided into 4 equal quadrants: 2 open areas without walls and 2 walled areas. Mice were placed in the closed area of the maze, and activity was recorded for 8 min by a video camera mounted above the maze. This test is performed under dim light (about 40 lux). Time spent in the open quadrants was later scored and indicated the level of state anxiety. To assess marble burying behavior, marbles were evenly spaced in a plastic cage with a 5-cm layer of bedding to which the animals had previously been habituated. Mice were placed in the cage and recorded by video camera for 20 minutes. At the end of the test, marbles that were more than 2/3 covered by bedding were considered "buried" and used as a measure of anxiety (Njung'e et al., 1991) . Videos from the test and habituations were scored for the time spent digging.

Tests of motor function

To test motor function, we used the rotarod test, climbing, and gait analysis. Rotarod was performed on an accelerating rod apparatus (Ugo Basile). For training, mice were run until all the animals stayed on for more than 200 sec (at least 3 training days). On subsequent testing days, animals were tested three times. In the untrained experiments, animals were run for three trials each testing day without any previous experience. To assess climbing behavior, animals were placed into a 40 cm high wire-mesh cylinder with a clear plastic top (Deacon et al., 2005).

Behavior was recorded by video for 10 minutes and time spent climbing, defined by all four limbs off the floor, was measured. Gait analysis was performed using the Noldus CatWalk system(Vrinten et al., 2003) . Briefly, mice were trained to walk across a clear glass runway. After they could move through the apparatus without pauses, tracks are recorded and analyzed using the CatWalk software.

Other affective tests

Other affective tests were performed on some groups of mice. Forced swim test of despair-like depression behavior was performed before and after injury. For this test, animals were placed in a large beaker of water (about 25°C), in which animals could not reach the bottom. Behavior was recorded for 6 minutes, after which, time spent immobile (defined as not actively swimming) was counted, a measure of the time in "despair"-like behavior (Porsolt et al., 1977) . As a second measure of depression-like behavior, we used the sucrose preference test to document the presence of anhedonia. Mice were given the choice of drinking water or 2% sucrose for two consecutive nights. The position of the water and sucrose bottles was switched on the second night. Mice were trained once before obtaining two baseline measures. A preference score was given as a percent of sucrose liquid consumed over two nights compared to total liquid consumed. In normal mice, this is generally above 90%. Anhedonia was considered to have occurred if preference dropped to 65% (Monleon et al., 1995; Strekalova et al., 2004). Time spent in social interaction was recorded in a new clean cage with a novel animal of similar size and

strain for 8 min. Social sniffing, exploration and physical contact were all counted as signs of interaction.

Experimental design and statistical analyses

Separate groups of animals were used for the home cage monitoring, motor behavior and affective behavior experiments. Supplemental figure 5 shows the timing and group distribution for HCM and affective behavior testing. Additional groups were used to test open field activity at days 3 or 7, followed by rotarod (untrained) and to test locomotor activity on the catwalk and rotarod (trained). Animals were allocated to groups in a block design, by the same researcher who also performed the surgeries and behavioral testing. No animals were excluded from the study once data were collected. After the surgeries, the identity of the cages was concealed from the researcher performing behavioral tests and not revealed until all data collection and analysis were completed. For animals in the home cage monitoring experiment, only the Von Frey testing before and after the monitoring period was done blind. As the monitoring system and subsequent analysis is automated, this part of the experiment was not blinded.

Results are expressed as mean±SEM and p values less than 0.05 were considered significant. Comparisons were analyzed with one-way or repeated measure analysis of variance (ANOVA). In experiments with only one control group, we used Student's t-test, except in cases where data were non-parametric, for which the

Mann-Whitney u-test was used. For ANOVAs, post-hoc analysis used Bonferroni tests. Data were analyzed using GraphPad Prism 5 for Mac.

Results

Mechanical hypersensitivity in different chronic pain models

With the exception of a few animals that underwent CCI or SNI and appeared to favor the uninjured hindlimb, gross observation of mice after injury rarely revealed notable signs of pain or discomfort in any of the injury models. Unquestionably, however, animals in each injury group had significant decreases in mechanical sensitivity (i.e. mechanical hypersensitivity) after injury (Fig. 1A and B). Mechanical withdrawal thresholds of naïve, saline and sham controls also decreased over time, albeit to a smaller extent. This drop might reflect the fact that animals undergo other tests during the post-surgical period, which might alter, to a small extent, their behavior in this test. The fact that mice were only tested weekly after injury, rather, that every other day for baseline testing, may also be relevant. However, regardless of the explanation, when data for the two nerve injury groups were normalized to the naïve control, both SNI and CCI groups still showed a significant decrease in threshold. Sham controls did not differ from naive at any time point (Fig. 1C). Nerve-injured and CFA-injected mice that underwent home cage monitoring were also tested for mechanical hypersensitivity before being introduced into the monitoring system. Animals in these injury groups all developed mechanical hypersensitivity, and this was still present when these mice were removed from the system after 16 days (Supplemental Figure 1A-D).

Monitoring of home cage activity

Animals were placed in the home cage monitoring (HCM) system on the second day after surgery or after CFA injection. The first day in the HCM is generally marked by increased activity, which is presumably a manifestation of the animal's exploration of the new cage and its building of a new nest. This increased exploration is expressed as an elevated distance traveled on day one in the HCM compared to subsequent days. In fact, all animals with SNI, CCI or CFA and their respective controls displayed this elevated locomotion. On the other hand, although CCI animals showed a normal heightened response to the novel environment (157% greater on day 1 compared to day 3 of monitoring for CCI, 175% in Naïve controls, $p=0.38$, Supplemental Figure 1F), their total distance traveled on the first day of monitoring (corresponding to day 2 after injury) was significantly less than naïve controls (naïve vs. CCI, 1110±85m vs. 605±36m, $F=17.5$, $p<0.0001$, Fig. 2A). Animals with SNI or CFA did not have a similar significant reduction in locomotion on the first monitoring day compared to control groups. Interestingly, animals with CCI sham injury, like their full surgery counterparts, moved significantly less on the first day of monitoring compared to naives and were not different from CCI animals (Fig. 2A). Animals that underwent sham SNI surgery did not differ in distance traveled from mice with SNI or from their naïve controls, indicating that the two sham surgeries are not equivalent, possibly as a result of the more proximal site of injury in the CCI group. The locomotor difference between CCI and naïve controls was also seen in the average daily movement over days 3-17 after injury, although

sham was not significantly different from either group. (Fig. 2B) Again, there was no difference between SNI or CFA and controls.

To observe better the activity differences among groups throughout the course of the monitoring period, we analyzed light and dark cycle locomotor activity separately, on each day (days 3-17 after injury.) There was no difference among animals with SNI, CFA injection, sham and naïve controls in the light and the dark cycle (Fig. 2Cc-h). In the CCI experiments, however, repeated measures ANOVA revealed a significant effect of treatment groups during both the light and dark cycles (dark, $F=6.8$, $p=0.0023$; light, $F= 4.02$, $p=0.0235$, Fig2Ca-b). Thus post hoc analysis of the dark cycle activity indicated a significant difference between CCI and naïve groups during the first 12 days post-injury; the sham group was only significantly different from the naïve group on days 8 and 9 and did not differ from CCI on any day. In contrast to the active phase of the mouse, light cycle activity (i.e. inactive phase) in CCI animals was only reduced in the first week post-injury. It appears therefore that SNI and CFA did not alter overall activity, but CCI caused a significant, albeit transient (for 12 days) decrease in daily activity.

SNI, CCI and CCI sham injuries all resulted in significant loss of weight after the first 2 days of injury (measured just before monitoring began.) Moreover, all groups had similar weights upon removal from the monitoring system, i.e. at day 17 post-injury. Thus, injury groups gained slightly more weight during the monitoring period, though not significantly so (Supplemental Figure 1E). However, despite the small

differences in weight gain/loss in these groups, there were no differences in average daily food and water intake among any experimental and control groups (Fig. 2D-E). Even during the early days after injury (day 3 to 7), a time when CCI animals moved considerably less than did the controls, the food and water intake of mice with CCI did not change (Supplemental Table 1).

Circadian patterns of activity in nerve-injured animals

The circadian patterns of the two strains used in this study are very stereotyped and reproducible. Both Balb/c and Bl/6 mice have a peak of activity immediately after the dark cycle onset, and the Bl/6 mice have a second peak of activity around the end of the dark cycle (Fig. 3A). Because we initially hypothesized that the circadian pattern of animals experiencing persistent pain would become more fragmented(Landis et al., 1988), we focused our analysis on the Balb/c strain, expecting to observe disruption of the extended peak of activity in the first half of the dark cycle.

A great advantage of the HCM system is that it allows for precise temporal analysis of the animal's activity over the course of the day. Because the decrease in activity in the CCI and sham groups only occurs in the early post-surgical period, we divided the circadian analysis into an early (days 3 to 7) and late (days 13 to 17) period. None of the injury groups displayed dramatic changes to the circadian pattern. In fact, for animals with SNI or CFA, there were no differences in distance traveled at any time of the day, in either the early or late monitoring period (Fig. 3Cc-d). As

expected, during the early analysis period, animals in the CCI group moved significantly less than did animals in the naïve group, during the first half of the dark cycle (effect of surgery, $F=14.5$, $p< 0.0001$; post-hoc tests between CCI and Naïve are significant from 7pm to 1am, Fig. 3Ca). For animals with CCI sham injury, the amount of dark cycle activity fell between CCI and naïve group levels, being significantly different from naïve for the first half of the dark cycle ($p<0.0001$, post-hoc differences at 7pm-12am) and from CCI during only two hours early in the dark cycle (post-hoc differences at 8 and 9pm). By the final days of monitoring, however, there were no longer any significant differences in the distance traveled at any time of day for the CCI experiment (effect of surgery, $F=3.1$, $p=0.0505$, Fig. 3Cb), indicating that CCI and to a lesser extent CCI sham surgeries, lead to an initial decrease in activity, which recovered by 2 weeks.

In addition to using distance traveled at various times of day as an indication of circadian pattern, the HCM system can further define the state of an animal (active or inactive), allowing detailed analysis of a mouse's daily life(Goulding et al., 2008) . We found that the animals' circadian rhythms did not change, using either a measure of distance traveled or the probability of being in an active state over circadian time. And, as when using distance moved, animals with CCI have a decreased active state probability during the dark cycle, but only early after injury (early, $F=6.1$, $p=0.006$; late, $F=0.01$, $p=0.99$, Supplemental Fig. 2A).

Bout and time budget analysis

Behavior within the active state can be further classified as feeding, drinking, moving or other (active, but not in one of the three main activities.) This is particularly useful as we had predicted that given the need to climb onto the feeder to eat, injured animals might have fewer, but longer feeding bouts. However, even in the early monitoring period, when there were changes in locomotion, animals with CCI had no significant difference in feeding bout number, size or duration. Nor was there a difference in the SNI or CFA groups (Supplemental table 1, 2). We did observe that movement bouts of animals with CCI were both fewer in number and shorter (in terms of distance per bout) during days 3 to 7, but this recovered by the later period of monitoring (Days 3-7: 4150 vs. 2710 bouts per day in naïve vs. CCI, p=0.0006; Days 13-17: 4183 vs. 3902, p=0.297; Supplemental Table 1). Importantly, movement bout averages were not different from controls in any other injured group. Taken together these data indicate that it is the combination of less time spent in the active state as well as a dramatic reduction in movement bouts during this state that likely contribute to the decrease in overall activity seen in animals with CCI in the first week of monitoring.

Examining the time budgets of the animals is particularly helpful for appreciating the differences in the CCI group of animals in early vs. late monitoring periods. To this end, we measured and compared time spent inactive, moving, feeding, drinking, or other (i.e. stopped, but active). While SNI and CFA did not alter any parameter of daily activity (Supplemental Figure 2 and Supplemental Table 2), CCI animals,

during the initial monitoring days, spent significantly less of the day engaged in locomotion (6.3% vs. 4.1%, p=0.0004) and more of the day inactive (59% vs. 67%, p=0.002, Fig. 3B). This difference was no longer present during the late monitoring period.

Note that although we focused our analysis on Balb/c animals, we also studied C57Bl/6 animals with SNI or sham and naïve controls. Much like Balb/c mice that underwent SNI, we found no changes in the movement and feeding bout properties, and time budgets of injured animals did not differ, when compared to either sham or naïve controls (Supplemental Figure 2 and Supplemental Table 2).

Other tests of daily life activity

Because animals with CCI displayed reduced locomotion in the home cage, we also tested separate groups of Balb/c mice in an open field test on days 3 or 7 after CCI, SNI or CCI sham surgery (naïve controls received anesthesia only). On neither day did animals with SNI show a decrease in distance traveled; and perhaps more surprisingly, this was also true for the mice with CCI (Fig. 4C, day 3 p=0.23, day 7 p=0.88). We also tested animals for open field activity one month after injury, in each of the pain models and in both Balb/c and C57Bl/6 mice. No pain model resulted in significant decreases in distance traveled (Fig. 4A,B).

As the open field only measures horizontal movement, we also examined vertical movement. In this test, the animals were allowed to explore a climbing apparatus, in

which they had an opportunity to climb on a wire mesh grid, an activity that requires the use of all 4 limbs. At 3 weeks post-injury, animals with SNI and CFA injections climbed for the same amount of time (10 min test) as did control animals (Fig. 4F). We also counted the number of rearing events in the elevated maze and found no difference between injured and control animals in any group. (Supplemental Fig. 3A)

As noted above, occasionally we observed animals in the nerve-injured groups that appeared to have an altered stance. Thus, to assay for possible motor deficits, we tested sham, SNI or CCI groups of mice on the accelerating rotarod. All animals were trained and performed equally well prior to surgery, and this did not change on days 4 to 11 after surgery (naïve, 109±8% baseline; SNI, 104±6%; CCI, 94±6%, p=0.62, Fig. 4D, Supplemental Figure 3B). Interestingly, however, when animals with no prior experience on the rotarod were tested 3 days after injury, those in the CCI and SNI groups stayed on the apparatus for a significantly shorter time than compared to untrained naïve controls (p= 0.0059, Fig. 4E). When these animals were retested on days 5 and 9 after surgery, the apparent motor deficit was still present (Supplemental Figure 3C). By contrast, when untrained animals had their first training day a full week post-injury, we found no significant difference among groups (Supplemental Figure 3D). Finally, we analyzed the locomotor ability of CCI injured animals using the CatWalk system, which allows for gait analysis. Even in this test, there were no changes in parameters of ipsilateral vs. contralateral hindpaw pressure or timing of the stride (Supplemental Figure 3E), indicating that

there is no change in the use of the injured paw when the animal is in motion. However, we cannot rule out alterations in weight distribution in stationary animals.

Behavioral tests of affective state

To determine if any of the pain models are associated with a change in affective state, we next performed a battery of behavioral tests of emotion. To assay for the level of anxiety, we used the open field, elevated maze and marble burying tests. At time points soon after surgery (days 3 and 7) and later (one month), we found little difference in the open field test of anxiety, i.e. time spent in the center area, among any of the injury groups and controls (Fig. 5A and Supplemental Fig. 4). Likewise, elevated zero maze time in the open areas revealed little difference among injury and control groups (Fig. 5B). On the other hand, although none of the injury groups showed consistent differences in either measures of anxiety, a few groups did show an unexpected decrease in anxiety-like behavior on one of the measures. Thus, Balb/c animals with SNI or CFA injury spent more time in the center area of the open field (SNI, $p= 0.0173$; CFA, $p= 0.0278$); and C57Bl/6 animals with CCI spent more time in the open areas of the elevated maze ($p= 0.0169$). In other words, while there was no consistent change in the two related measures, these injured animals may, if anything, have reduced anxiety. However, we do not rule out the possibility that there was a floor effect in the naïve animals, especially in the case of Balb/c mice, which prevented us from observing any further changes in injured animals.

Additionally, we observed no difference in anxiety-like behavior in the marble burying test. Thus, when tested at both 5 and 7 weeks after SNI surgery, C57Bl/6 animals buried the same number of marbles as did controls. And Balb/c mice with CCI or CFA injection did not display any difference in anxiety state in the marble-burying task (Fig. 5C). Furthermore, in the testing cage, we found no difference in the time the injured groups of mice spent digging compared to controls (data not shown), with or without the presence of the marbles. So even using a different test of anxiety, injured animals showed no change in their anxiety-like state.

Although anxiety was the focus of the affective state behavior tests, we also tested some groups of animals using the forced swim test of depression-like behavior, sucrose preference test of anhedonia and a social interaction test (Supplemental Fig. 4). In the forced swim test, neither CFA nor SNI significantly altered the amount of time animals spent immobile, i.e. we found no evidence for an increased depressive state. The same conclusion was drawn in the sucrose preference test, where C57Bl/6 animals with SNI or CFA, drank similar amounts of sweetened water compared to their respective sham or saline injected controls and never fell below 65% preference. Lastly, we found that there was no change in the time spent exploring a novel mouse, which is presumed to test a more complex affective state. Here too, injury did not appear to interfere with normal social interaction.

Open field behavior after short-term persistent pain

Having found that our standard models of chronic pain did not alter behavior in tests of locomotion and affective behavior, we also tested animals in a shorter duration persistent pain model in the open field test. We used a model of pain that is associated with very overt signs of discomfort (compared to that observed in CFA-injected animals). In these tests, C57Bl/6 and Balb/c mice received intraplantar injections of formalin and were tested two hours later. Because we did not want to confound the analysis during the time the animal licked its hindpaw in response to the formalin, we performed the open field test in the period immediately after licking behavior had ended. As for the longer-term persistent pain models, we again found no difference in the overall distance traveled in the open field, for either strain (Fig. 6A, B). And while there was no significant difference between saline- and formalin-injected animals in the time spent in the anxiety-related center area, Balb/c animals with formalin did show a trend to less time in the center, i.e. appeared to display more anxiety (Fig. 6C, D).

Finally, as a positive control for the open field experiments, we also tested animals two hours after sickness was induced with an injection of LPS. Animals with LPS were fully capable of ambulating, as they reacted by running from the researchers hand as did control mice. In the open field, however, LPS-injected mice showed a profound reduction in both distance traveled and time spent in the center, indicating an increased anxiety state and an overall decrease in activity (Fig. 6).

Since the lack of movement likely confounds activity measured in the center, when we normalized the distance in the center to the total distance moved, we also found that C57Bl/6 animals with LPS still showed significant anxiety-like behavior (5% vs. 0.8%, data not shown). We conclude that the test is sufficiently sensitive to detect changes in behaviors after sickness, but that pain alone is not sufficient to reveal differences.

Discussion

Humans with chronic pain often have higher measures of depression and anxiety (Geisser et al., 2000; Price, 2000) and score lower on quality of life inventories (Liedberg et al., 2009), which typically measure the impact of pain on daily activity, overall mood, sleep, social function, etc. (O'Connor, 2009). Here we addressed the behavioral impact of persistent pain in animals by monitoring the daily life of a mouse using three different standard mouse models of chronic pain. We also studied the mice using a battery of tests of affective state. In the home cage, we found that none of the injury models had a lasting impact on basic parameters of daily life activity, such as daily food intake and locomotion. Only mice with CCI showed an early, but short-lived decrease of activity and only this group had significant alterations in other patterns of home cage daily life (e.g. increased time spent in the inactive state.) All groups showed similar behavior in tests of affective state. Taken together, these results indicate that despite the profound and prolonged mechanical hypersensitivity characteristic of these different "pain" models (inflammatory and neuropathic), there is minimal to no alteration in what

we define as quality of life measures. Our results suggest that these standard models, at least when used in the mouse, do not adequately incorporate important features of the human chronic pain condition, raising questions as to whether mice experience significant ongoing pain with these injuries.

Home cage behavior is altered only early after CCI

Despite the findings of early postoperative changes in the CCI group, we found no changes in the SNI group of mice, even though the two nerve injury models are presumed to have similar etiology and both are associated with mechanical hypersensitivity. Comparison of results with sham-treated animals suggests that much of the difference is attributable to differences in the surgical procedures. For example, incisions for the CCI procedure are more proximal, which might be more disruptive to the animals. In effect, the initial decrease seen in the CCI groups could reflect differences in the time to fully recover from the surgery. On the other hand, neither sham group showed a significant development of hypersensitivity compared to naïve animals, demonstrating an important dissociation between the level of hypersensitivity and daily behavior in the home cage.

Somewhat surprisingly, however, the short-term reduction in home cage behavior in animals with CCI was not recapitulated in short-duration locomotor tests performed during the same time window. Indeed, we found very little motor deficit in either nerve injury group, despite the large denervation that occurs. This discrepancy suggests that the HCM may be more sensitive for detecting pain-related

behavior than are these short duration tests of locomotor activity. Conceivably, decreases in home cage activity result from a summation of many small bouts of spontaneous pain, which the shorter open field tests miss. Contrary to this explanation, however, mice with persistent pain caused by intraplantar formalin, where there are measurable signs of discomfort, also did not alter activity in the open field. It is also possible that the HCM, which uses a more normal, familiar, environment, facilitates detection of behavioral differences that are otherwise masked by the novelty of the open field test.

There are, however, two limitations of the HCM method that merit discussion. First, we cannot directly measure sleep, disruption of which is an often-reported problem in chronic pain patients (Pilowsky et al., 1985) and has been observed in some studies of rats with nerve injury or experimental arthritis (Guevara-López et al., 2009; Landis et al., 1988; Monassi et al., 2003; Tokunaga et al., 2007). By documenting periods and patterns of inactivity, the HCM system can, to some extent, provide a reasonable estimate of sleep. However, we have no information as to possible disruptions of sleep architecture. A second major limitation of the HCM is that the mice must be individually housed. Although our short-duration social interaction tests showed no differences, there could be significant persistent pain-associated disruption of normal social interaction among cage mates. Indeed, it is our impression that immediately after surgery, injured animals, in both the sham and nerve injury groups, sleep apart, not huddling, as do intact mice.

Alterations of affective state in standard chronic pain models

Previous studies using persistent pain models found conflicting results as to whether injured mice show changes in anxiety- and depression-like behaviors (Benbouzid et al., 2008; Hasnie et al., 2007b; Narita et al., 2006a; Suzuki et al., 2007). In the present study, we found that mice in the SNI, CCI or CFA groups did not differ in measures of affective state using an array of tests; this was true for weeks after injury. If anything, the few significant differences detected were all opposite to what we initially predicted, given previous studies in the rat. That is, mice appeared less, rather than more anxious. However, these few changes were neither consistent across all tests of anxiety state nor across mouse strain. Indeed, our results fit well with a previous study in mice that reported that nerve injury did not alter open field or elevated plus maze behavior at 1, 2 or 4 weeks after injury(Hasnie et al., 2007b). Moreover, our results are consistent between strain and injury type as well as with the absence of a long-term change in home cage behavior.

In contrast to our findings, there is a sizable body of work on rats showing changes in anxiety- and depression-like behaviors occurring after nerve injury and inflammation (Gonçalves et al., 2008; Hasnie et al., 2007a; Hu et al., 2009; Ji et al., 2007; Kontinen et al., 1999; Leite-Almeida et al., 2009; Roeska et al., 2008). If CFA, CCI or SNI in mice produces pain that models the full human condition, or even replicates the behavior of a rat, we would expect an impact on the emotional state of a mouse. Our results could be due to the particular methods that we used or to the species itself. As a prey animal, mice do not show signs of weakness, including overt

signs of persistent pain. Regarding the latter, it is of interest that we did observe changes in the LPS injected animal, indicating that mice have the capacity to display sickness behavior. There are, of course, other examples of rat behaviors do not translate to the mouse, such as the positive and negative affect of the different vocalization frequencies in the rat (Knutson et al., 2002; Portfors, 2007). Finally, as some of these post-injury changes are age (Leite-Almeida et al., 2009) and gender (Koehl et al., 2006; Mogil, 2009; Painsipp et al., 2007) dependent, it is also possible that our failure to replicate the rat data is simply a consequence of our focus on adult male mice.

These standard tests of affective behavior have two key problems in their use in pain studies. First, as for tests of mechanical hypersensitivity, the tests of emotional state are provocative, i.e. require an external stimulus. Evoked behavior might alter or mask the presence of spontaneous pain. This further highlights the importance of measuring daily home cage behavior, which, as a passive observation, is minimally disruptive of the animal's life. The second limitation stems from the validity of these tests themselves. Indeed, there has been extensive discussion in the affective science literature on the use of open field, elevated maze and forced swim test to reliably assess "anxiety" and "depression" (Cryan et al., 2005; Prut et al., 2003; Ramos, 2008).

Dissociation of mechanical hypersensitivity and quality of life measures

The most common endpoint in pre-clinical trials in rodent chronic pain models is evoked hypersensitivity. At present there are few other surrogates for chronic pain in rodents. The failure of some drugs in clinical trials (after extensive preclinical validation) may thus reflect the limitation of hypersensitivity as the only endpoint for monitoring of pain. Although hypersensitivity measures may be very useful for understanding mechanisms of nociception and defining potential targets for treating hypersensitivity, they are likely inadequate for the study of persistent, spontaneous pain. Indeed, by their very nature, measures of evoked hypersensitivity ignore the emotional and spontaneous components of the human chronic pain experience. Only operant models have successfully addressed the emotional component, albeit still an evoked response. Here we found a complete dissociation of hypersensitivity and quality of life measures. The mechanisms that drive the development of hypersensitivity, therefore, might be independent or simply insufficiently severe to drive changes in the daily life of a mouse.

As noted above, behavioral manifestations of quality of life changes have, to some extent, been demonstrated in the rat, but, given the value of genetic manipulation, the lack of a quality of life surrogate measure of pain in mice is unfortunate. In fact, our results raise the possibility that mice do not experience the persistent, ongoing pain that would affect what we have defined as quality of life measures. Indeed, a recent study reported that CCI did not alter behavior in short-duration tests of locomotion nor in any measurable outward sign of pain when observed at 2 or 4

weeks after injury (Mogil et al., 2010). Our data, along with this report, suggest that in these commonly used models of "chronic pain" there are no reliable measures of spontaneous pain even if the animal does experience it. If we are to continue to use mice in chronic pain studies, it is clearly essential that we develop new pain models that more accurately mimic the key characteristics of chronic pain experienced by humans.

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Figure 1

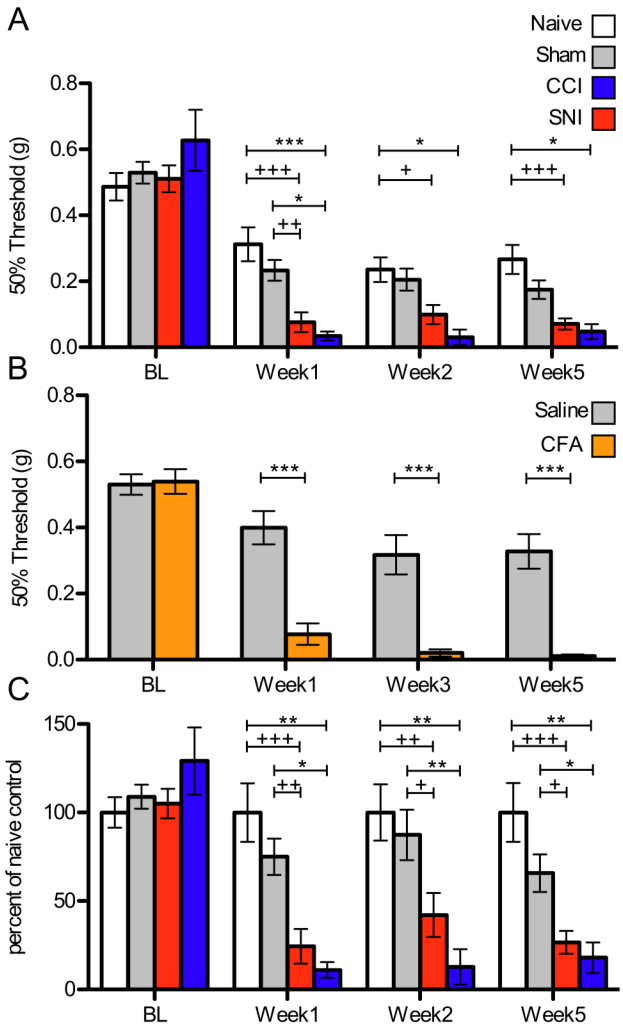

A
Naive
Sham
CCI
SNI
50% Threshold (g)
0.8
0.6
0.4
0.2
0.0
***
+++
*
++
*
+
*
+++
BL
Week1
Week2
Week5
B
Saline
CFA
50% Threshold (g)
0.8
0.6
0.4
0.2
0.0
***
***
***
BL
Week1
Week3
Week5
C
percent of naive control
150
100
50
0
**
+++
*
++
**
++
**
+
**
+++
*
+
BL
Week1
Week2
Week5

Figure 1. Mechanical hypersensitivity after nerve injury or inflammation. 50% withdrawal thresholds (in grams) were obtained using the von Frey up-down method in mice (A) after SNI (red bars) or CCI (blue), and in sham (grey) and naïve (white) controls or (B) after saline (grey) or CFA (orange) injection. (C) Mechanical thresholds were normalized to the naïve groups for the SNI and CCI experiments. For all, repeated measures ANOVA showed a significant effect of treatment with Bonferroni post-test differences between naïve/sham and CCI groups indicated with: * <0.05, ** <0.01, *** <0.001; post-hoc differences between naïve/sham and SNI groups: + <0.05, ++ <0.01, +++ <0.001

Figure 2.

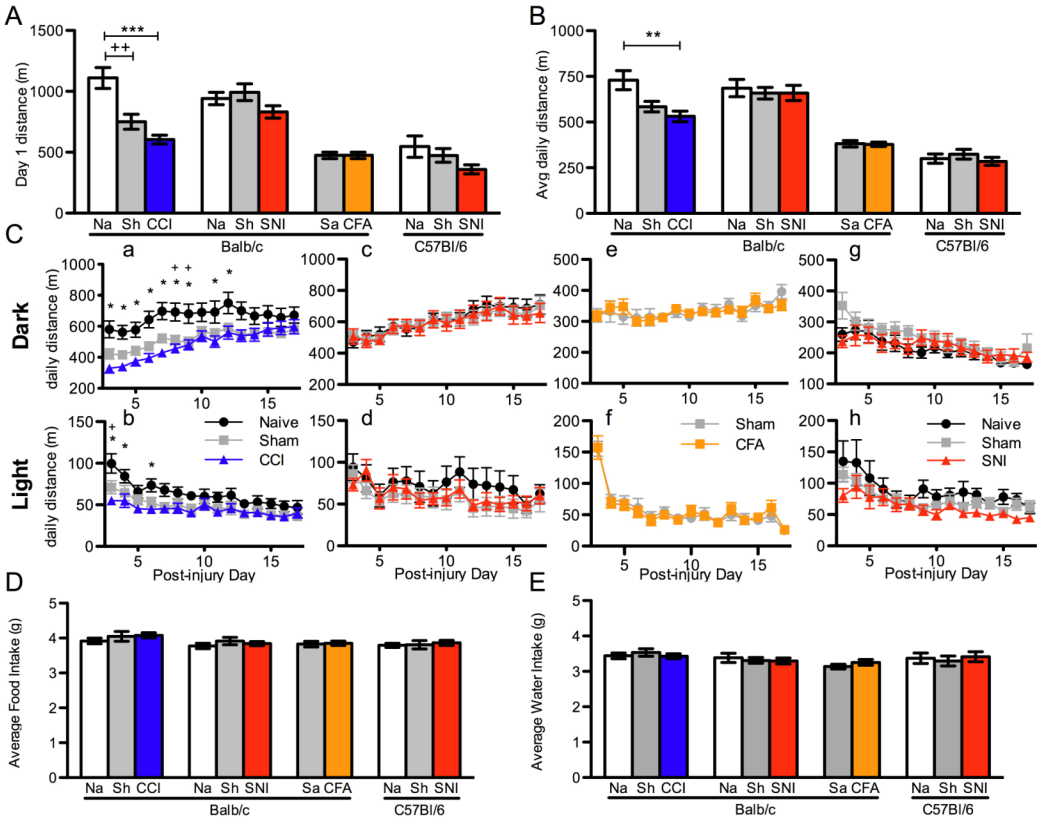

A
Day 1 distance (m)
Na Sh CCI
Na Sh SNI
Sa CFA
Na Sh SNI
Balb/c
C57Bl/6
B
Avg daily distance (m)
C
Dark
Light
daily distance (m)
Naive
Sham
CCI
CFA
SNI
Post-injury Day
D
Average Food Intake (g)
E
Average Water Intake (g)

Figure 2. Home cage movement, feeding and drinking in mice with SNI, CCI and CFA. (A) Total movement (in meters traveled) on day 1 of monitoring was significantly decreased in Balb/c mice with CCI (blue) and sham (grey) compared to naïve (white) controls, but not with either SNI (red) strain or Balb CFA (orange). (B) Average total movement over days 3-17 after injury was also significantly decreased in mice with CCI, but was similar to controls in all other experimental and sham groups. (C) Total daily movement in the dark (i.e. night; a, c, e, g) and light (b, d, f, h) cycles over days 3 to 17 was very similar to controls across the entire period for Balb/c mice with SNI (c, d) and CFA (e, f) and C57Bl/6 with SNI (g, h), but was significantly decreased in the early days after injury in the light cycle (b; repeated measures ANOVA, $p=0.024$) and for a longer period in the dark cycle (a; repeated measures ANOVA, $p=0.002$). Post-test differences are all indicated as less than 0.05 for ease of reading, though many are much smaller. Average daily food (D) and water (E) intake were no different in any group. Na: Naïve control; Sh: Sham control; Sa: Saline control. Post-test differences between CCI and Naive: *; differences between Sham and Naïve: +.

Figure 3.

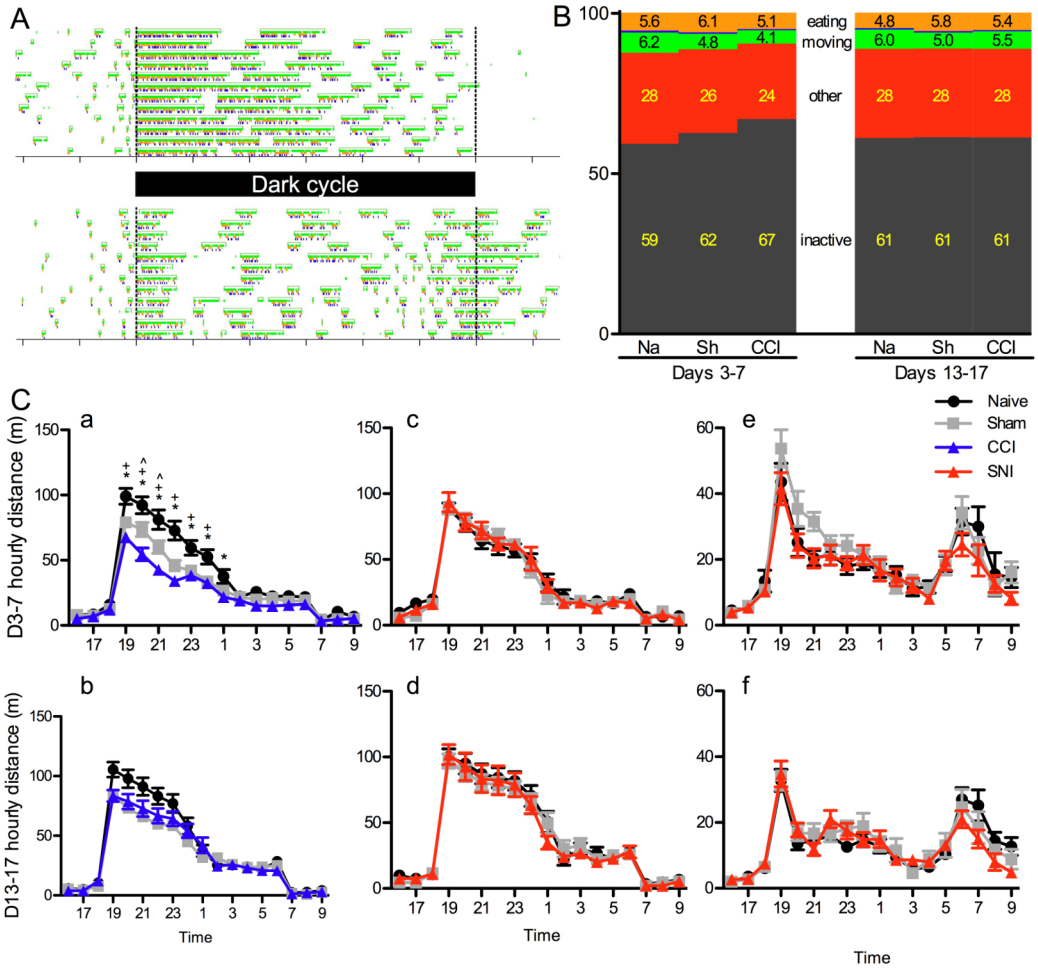

A
Dark cycle
B
100
50
0
5.6
6.1
5.1
6.2
4.8
4.1
28
26
24
59
62
67
eating
moving
other
inactive
4.8
5.8
5.4
6.0
5.0
5.5
28
28
28
61
61
61
Na
Sh
CCI
Days 3-7
Days 13-17
C
a
b
c
d
e
f
Naive
Sham
CCI
SNI
D3-7 hourly distance (m)
D13-17 hourly distance (m)
Time

Figure 3. Average distance traveled over circadian time. (A) Raster plots of a naïve mouse activity in Balb/c (top) and C57Bl/6 (bottom) mice over 12 days, with green representing periods of locomotor activity; orange, eating; and blue, drinking. (B) Time budgets of naïve, sham and CCI groups of mice in the early and late monitoring periods. Mouse time budgets include inactive (grey), locomotion (green), eating (orange), drinking (blue, less than 1% of the day, so not visible in the graph), and "other" (red, mouse is active, but not engaged in other activities). Inset numbers are percent of time in each activity. (C) Average movement (in distance traveled) binned by hour on days 3-7 (a, c, e) or days 13-17 (b, d, f). SNI Balb/c (c-d) and C57Bl/6 (e-f) animals did not show any differences in their circadian pattern in either the early or late days of the experiment. Average hourly distance traveled in the initial monitoring days by animals with CCI were significantly decreased from controls during the first half of the dark cycle (a; $p< 0.0001$), but not significantly different in the later days of monitoring (b; $p=0.0505$).

Figure 4.

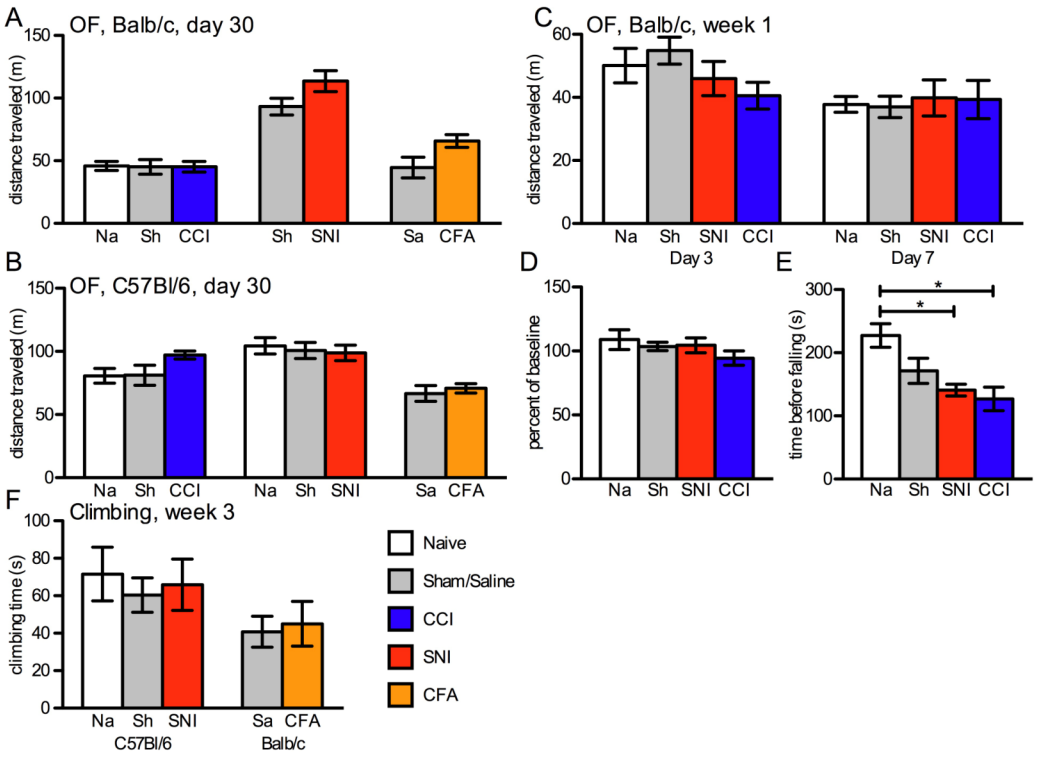

A
OF, Balb/c, day 30
distance traveled (m)
150
100
50
0
Na Sh CCI
Sh SNI
Sa CFA
B
OF, C57Bl/6, day 30
distance traveled (m)
150
100
50
0
Na Sh CCI
Na Sh SNI
Sa CFA
C
OF, Balb/c, week 1
distance traveled (m)
60
40
20
0
Na Sh SNI CCI
Day 3
Na Sh SNI CCI
Day 7
D
percent of baseline
150
100
50
0
Na Sh SNI CCI
E
time before falling (s)
300
200
100
0
*
*
Na Sh SNI CCI
F
Climbing, week 3
climbing time (s)
100
80
60
40
20
0
Na Sh SNI
C57Bl/6
Sa CFA
Balb/c
Naive
Sham/Saline
CCI
SNI
CFA

Figure 4. Short-duration tests of daily activity: (A) Distance traveled in the open field one month after injury was not different in any pain model in Balb/c mice (CCI n=8-9, p=0.99; SNI n=11-12, p=0.12; CFA n=6-7, p=0.07) or (B) Bl/6 mice (CCI n=8-9, p=0.09; SNI n=18-19, p=0.82; CFA n=8-9, p=1.0). (C) Distance traveled in the open field on days 3 and 7 after surgery did not differ among any of the experimental and control groups (n=8, Day 3 p=0.23, Day 7 p=0.97). (D) Mice with SNI or CCI performed as well after injury as before on the rotarod test when previously trained (n=5, p=0.62), (E) but significantly worse 3 days after surgery (n=8, p=0.006), if untrained. (E) Time spent climbing at 3 weeks after injury in Bl/6 mice with SNI or Balb/c mice with CFA did not differ from controls (SNI n=7-8, p=0.82; CFA n=10, p=1.0).

Figure 5.

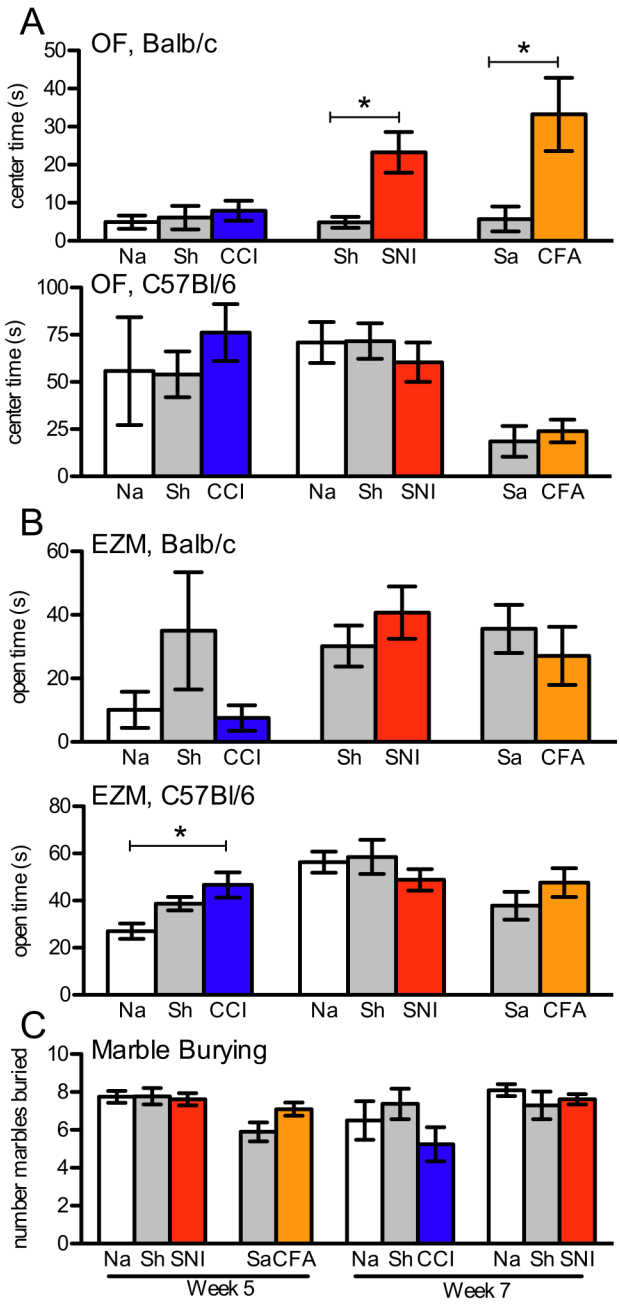

A
OF, Balb/c
center time (s)
50
40
30
20
10
0
*
*
Na Sh CCI Sh SNI Sa CFA
OF, C57Bl/6
center time (s)
100
75
50
25
0
Na Sh CCI Na Sh SNI Sa CFA
B
EZM, Balb/c
open time (s)
60
40
20
0
Na Sh CCI Sh SNI Sa CFA
EZM, C57Bl/6
open time (s)
80
60
40
20
0
*
Na Sh CCI Na Sh SNI Sa CFA
C
Marble Burying
number marbles buried
10
8
6
4
2
0
Na Sh SNI SaCFA Na Sh CCI Na Sh SNI
Week 5
Week 7

Figure 5. Behavioral measures of affective state at one month after injury. (A) Time spent in the center area of the open field in C57Bl/6 mice (bottom) with CCI (n=8-9, p=0.67), SNI (n=18-19, p=0.69) and CFA (n=8-9, p=0.58) was not different from controls. Balb/c animals (top) with CCI (n=8-9, p=0.71) did not differ from controls, but there was a significant increase in the time spent in the center in Balb/c mice with SNI (n=11-12, p=0.017) and CFA (n=6-7, p=0.073). (B) There was also little difference between experimental groups and controls in the time spent in the open areas of the elevated zero maze, in Balb/c (top) animals (CCI p=0.18, SNI p=0.33, CFA p=0.48) and in C57Bl/6 (bottom) animals (SNI p=0.44, CFA p=0.58) except for those with CCI that spent significantly more time in the open areas (p=0.017). (C) The number of marbles buried on week 5 in Bl/6 SNI animals or Balb CFA animals did not differ from control nor did Balb CCI or Bl/6 SNI differ from their controls at week 7.

Figure 6.

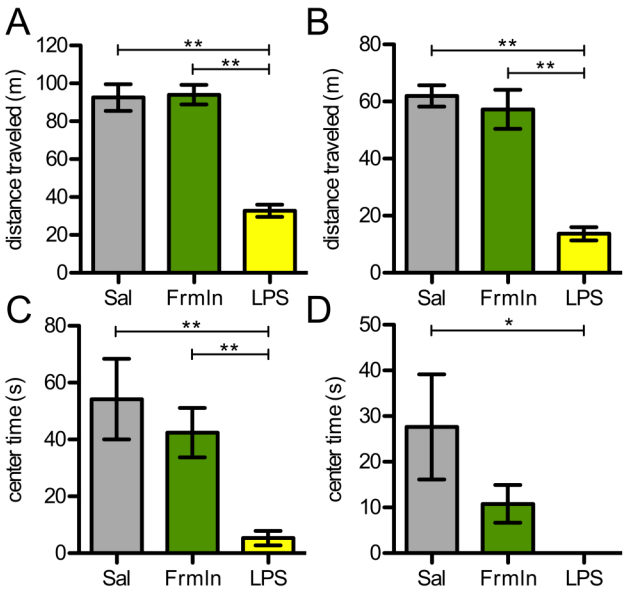
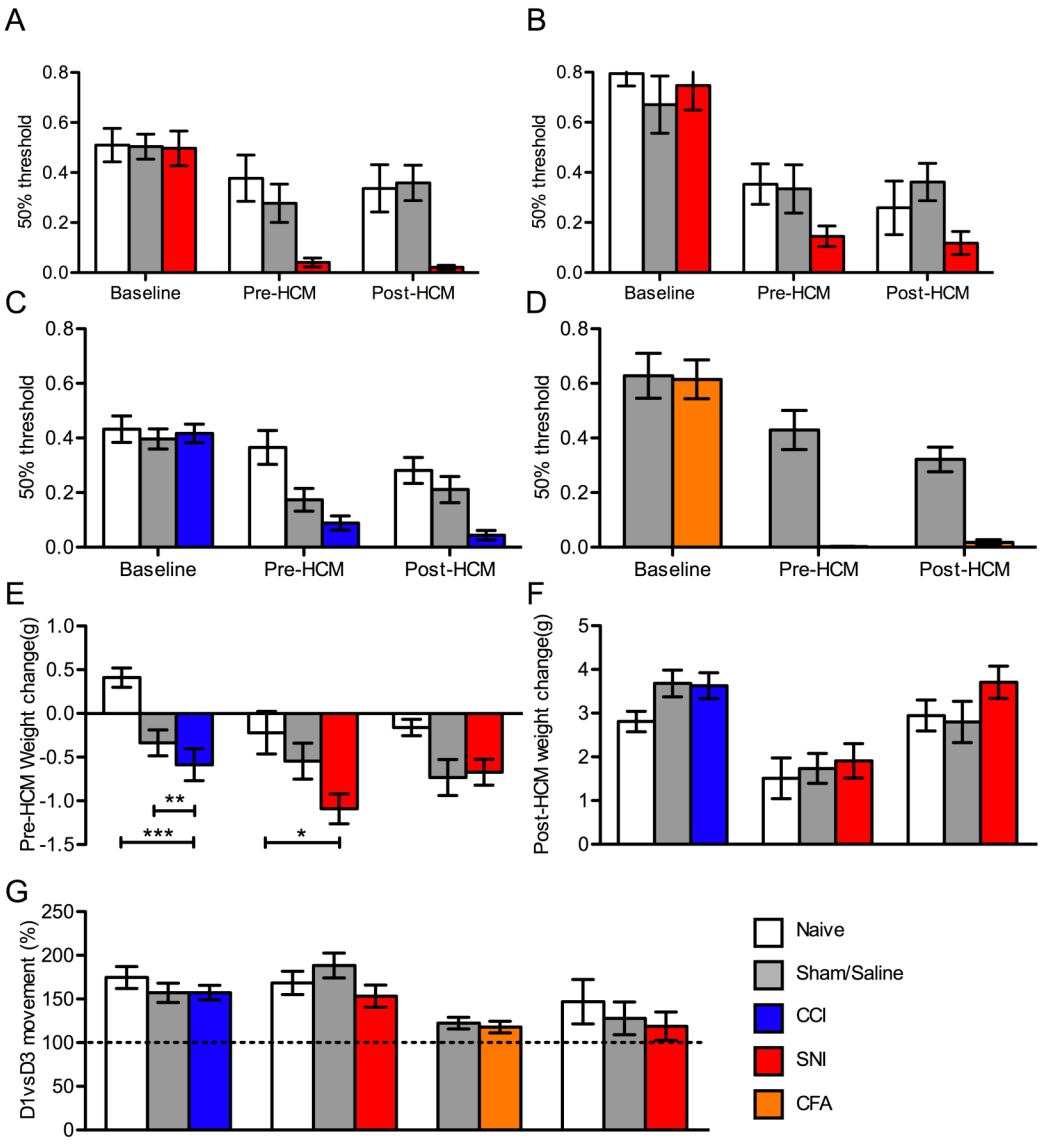


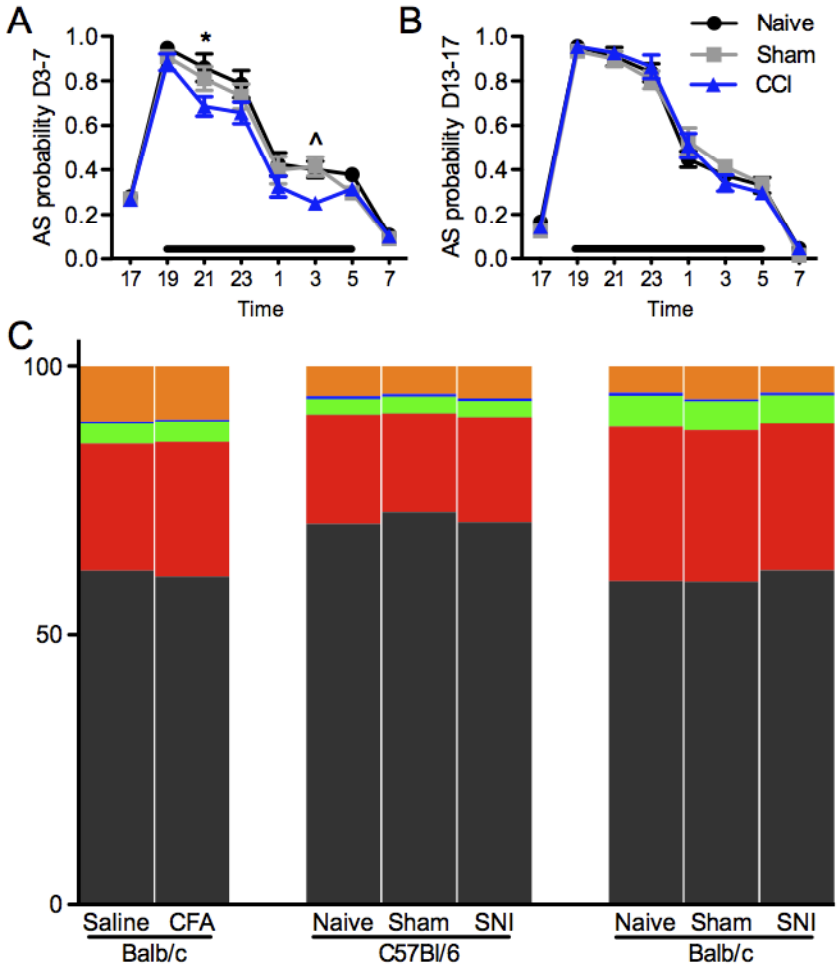
Figure 6. Open field behavior in short-term models of sickness and pain. (A-B) Distance traveled in the 30-minute open field test 2 hours after injection was severely decreased in animals injected with LPS, but not in those with intraplantar formalin in either (A) C57Bl/6 or (B) Balb/c animals (n=8/grp). (C-D) Sick animals also spent very little time in the center area compared to saline injected animals in (C) Bl/6 or (D) Balb/c strains. While formalin injected Balb/c animals showed a trend to less time in the center, the difference was not statistically significant (p=0.1). Sal: saline control; Frmln: formalin-injected group.

Supplemental Figure 1



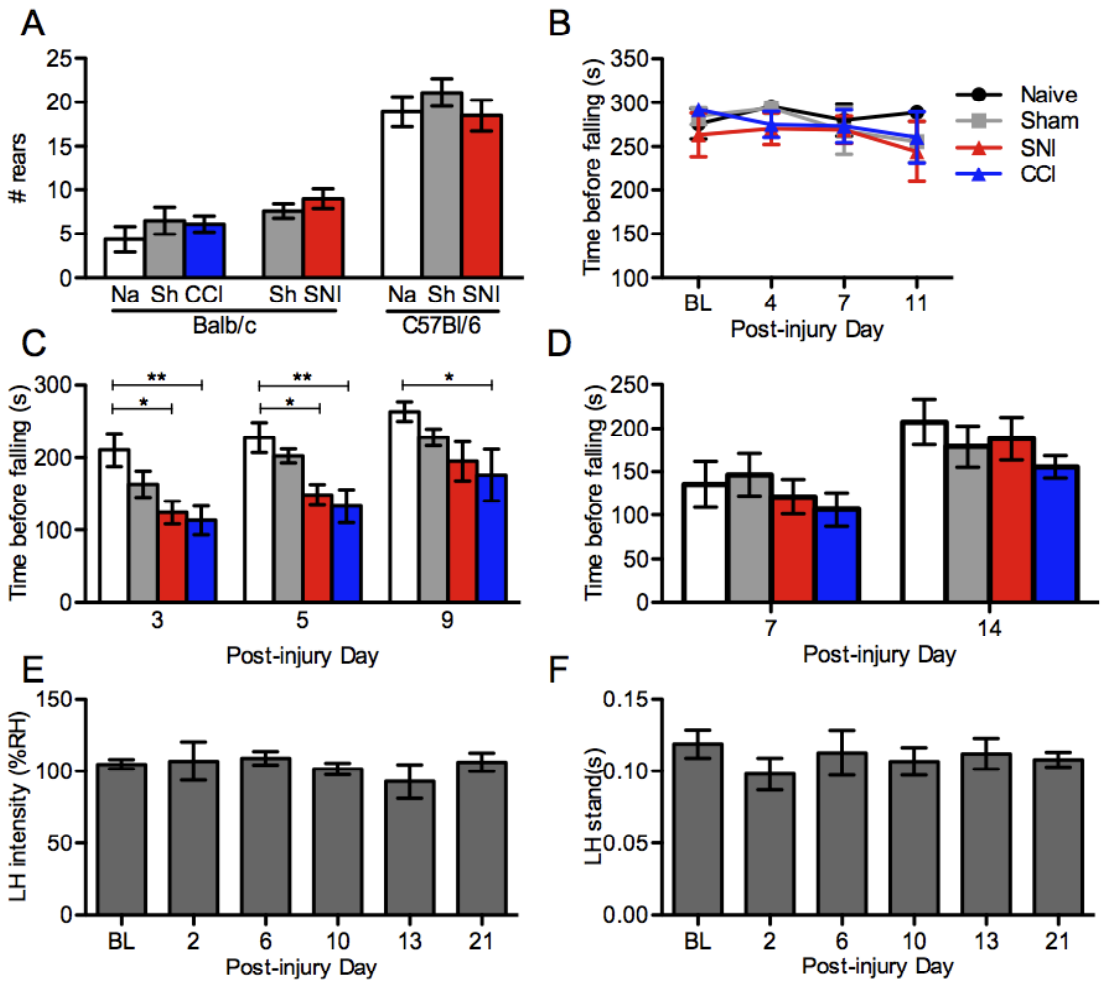
Supplemental Figure 1. Mechanical hypersensitivity developed in all groups of mice that were followed in the home cage monitoring (HCM) experiments: Balb/c with CCI (C), CFA (D) or SNI (A) and C57Bl/6 with SNI (B). Pre-HCM measures were taken the day after injury and the day before the start of monitoring. Post-HCM measurements were taken the day monitoring ended. (E) Balb/c animals with nerve injury lost significantly more weight during the first two days after injury; C57Bl/6 animals with SNI had no significant weight loss ($p=0.22$). (F) There was no significant difference in the amount of weight gained during the 16 days of monitoring (G) The first day of monitoring revealed an elevated activity compared to subsequent days; this was true for all groups of animals.

Supplemental Figure 2.



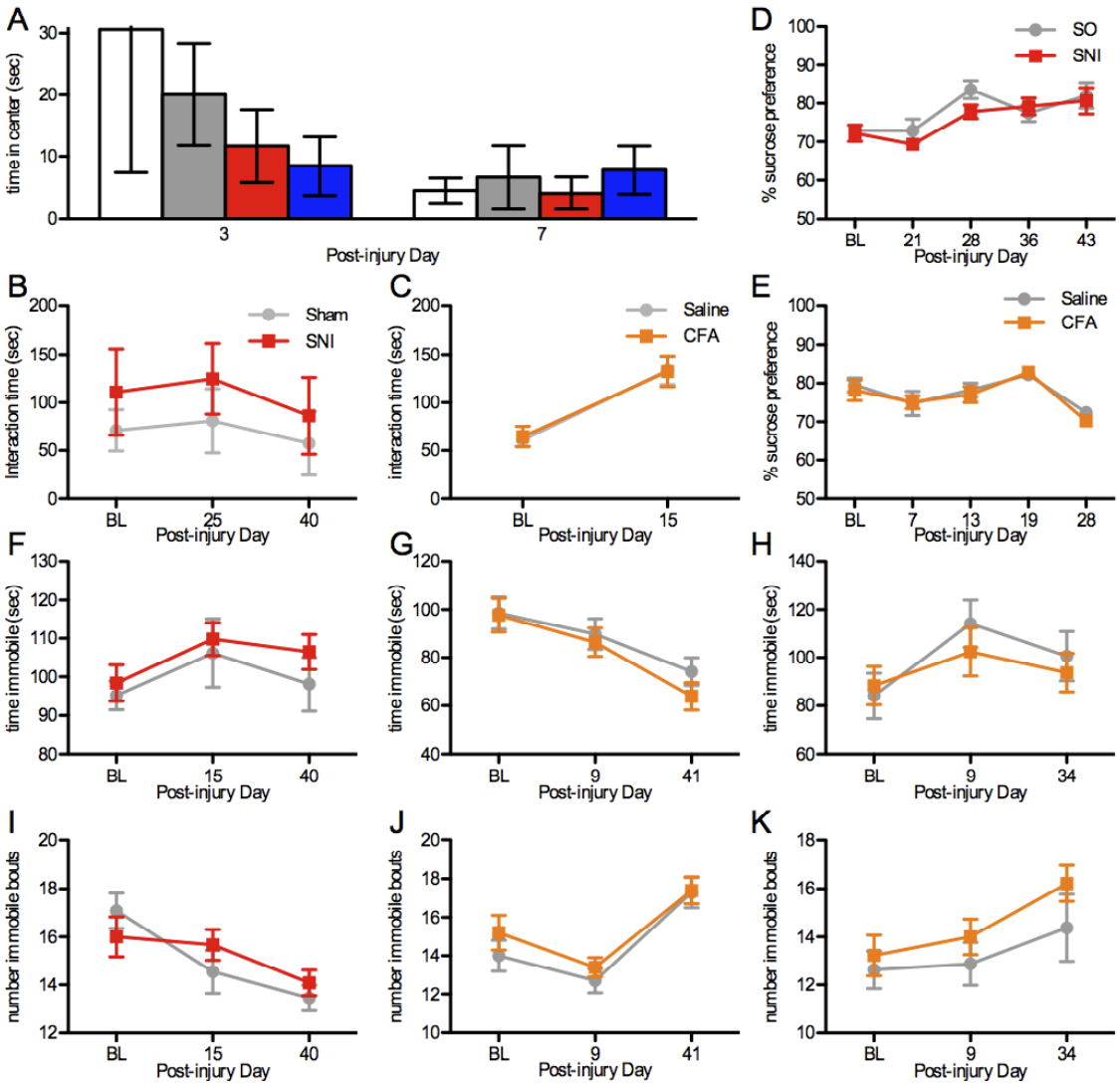
Supplemental Figure 2. Home cage monitoring circadian and budgeting patterns. (A-B) Probability of being in the active state (AS) over circadian time on days 3-7 (A) and 13-17 (B). Using an algorithm developed for the system, each animals' behavior was categorized as either active or inactive. Then the probability that an animal is active in each 2-hour time bin was calculated; black bar represents the dark cycle. (* $p<0.05$ Naïve vs. CCI, ^ $p<0.05$ Sham vs. CCI). (C) Time budgets from SNI and CFA HCM experiments. Grey: inactive, red: active, but not moving, green: moving, blue: drinking, and orange: feeding.

Supplemental Figure 3.



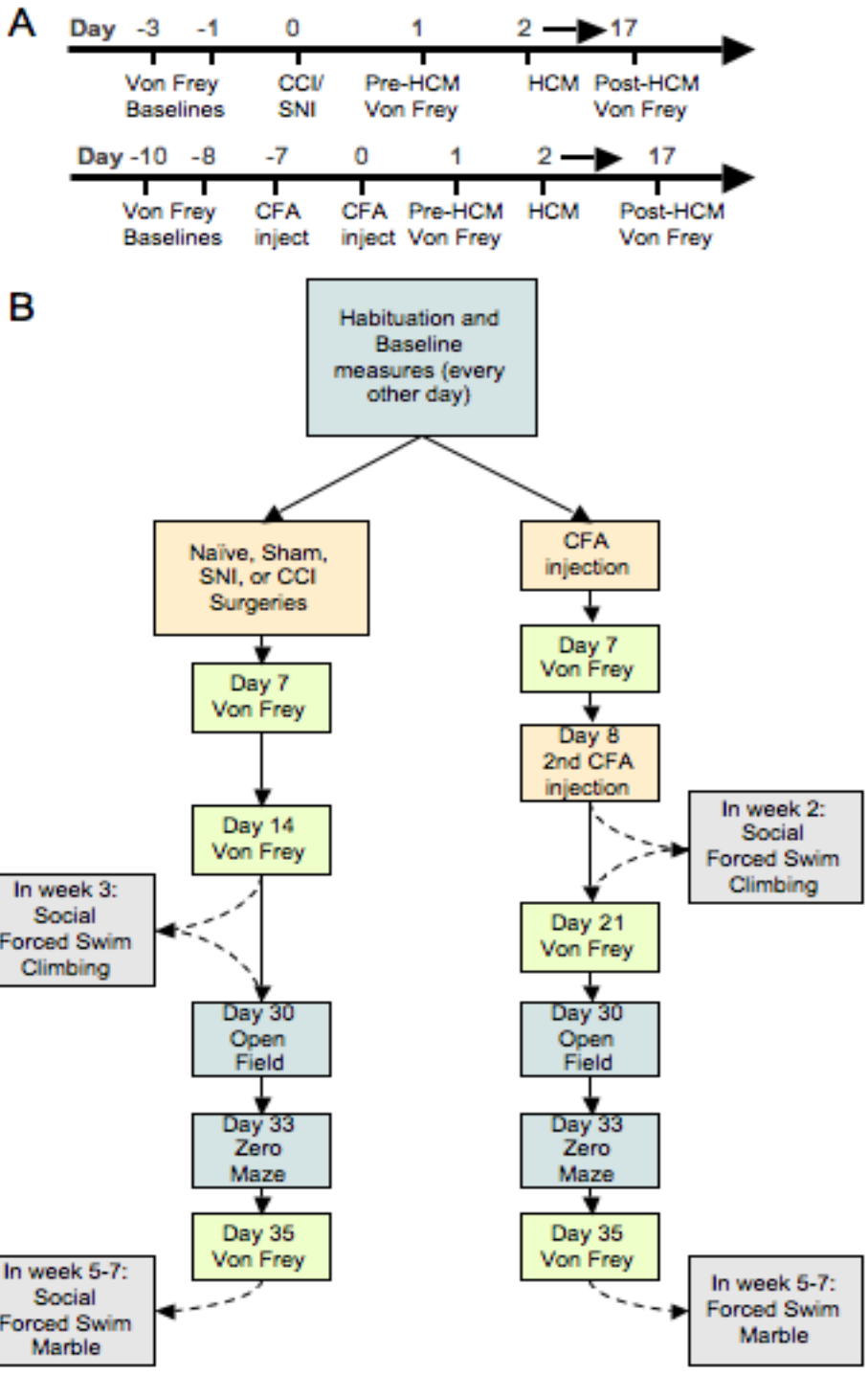
Supplemental Figure 3. Other tests of daily activity. (A) The number of rearing events in the closed areas during the 8 minute elevated zero maze test did not differ from controls in Balb/c animals with CCI or SNI or in Bl/6 animals with SNI. (B) Balb/c animals previously trained on the rotarod had no change in performance after CCI or SNI. (C) When Balb/c animals with CCI and SNI were not trained before being placed on the rotarod, they performed more poorly than did untrained controls. This was true for day 3 and on subsequent test days. (n=4, p=0.007) (D) However, when Balb/c animals with SNI or CCI had the first training day on day 7 after injury, there was no measurable deficit on the first or second day of rotarod testing. (n=4, p=0.6). (E-F) CCI animals did not change their gait on the CatWalk system, as measured either by analysis of the pressure put onto the ipsilateral hindpaw (E), or the length of time standing on the ipsilateral paw (F). BL indicates average baseline measurements.

Supplemental Figure 4.



Supplemental Figure 4. Measures of affective state. (A) There were no significant changes in anxiety-like behavior in the open field on days 3 or 7 (n=8). (B-C) Time spent in social interaction was also unchanged in C57Bl/6 with SNI (B; n=5) or CFA (C; n=8-9). (D-E) Preference for drinking sucrose did not decrease in C57Bl/6 animals with SNI (D; n=5) or CFA (E; n=8-9). Time spent immobile (F-H) and number of immobile bouts (I-K) in the forced swim test of despair-like behavior was not altered after SNI in Balb/c animals (F, I; n=11-12) nor after CFA in Balb/c (G, J; n=10) and C57Bl/6 (H, K; n=8-9) animals. BL indicates average baseline measurements.

Supplemental Figure 5



Supplemental Figure 5. Outline of experimental timing in (A) home cage monitoring experiment and (B) battery of affective behaviors. Grey boxes in timeline are tests that were not performed in every group of animals.

Supplemental Table 1

		Early Days 3-7				Late Days 13-17			
		Naïve	Sham	CCI	p	Naïve	Sham	CCI	p
Daily Avgs									
	24 hr. Food Intake, g	4.09±0.06	4.18±0.08	4.13±0.08	0.6979	3.79±0.08	3.83±0.07	3.93±0.07	0.36
	24 hr. Water Intake, g	3.55±0.10	3.75±0.12	3.75±0.12	0.3863	3.44±0.06	3.41±0.06	3.45±0.06	0.9123
	24 hr. Distance, m	699.5±50	528.1±27	422.8±24	< 0.0001; +,*	725.2±54	615.8±29	619.3±41	0.1289
	DC distance, m	619.6±46	470.2±26	373.4±24	< 0.0001; +,*	675.3±51	573.2±29	572.1±38	0.1253
	LC distance, m	79.94±7.4	57.97±4.8	49.48±4.4	0.001; +,*	49.88±5.8	42.46±4.5	43.32±6.2	0.596
Time Budgets									
	Inactive, %	59.15±1.5	62.45±1.2	66.85±1.4	0.002; *	60.90±0.9	61.17±1.0	61.07±1.1	0.9829
	Stopped, %	28.39±1.2	26.13±1.0	23.55±0.9	0.0101; *	27.84±0.5	27.70±0.8	27.69±0.8	0.9872
	Locomotion, %	6.28±0.49	4.82±0.31	4.09±0.20	0.0004; *	5.98±0.68	4.97±0.18	5.46±0.39	0.2979
	Feeding, %	5.60±0.31	6.14±0.32	5.08±0.44	0.1315	4.82±0.26	5.75±0.62	5.42±0.43	0.3852
	Drinking, %	0.58±0.13	0.46±0.02	0.43±0.03	0.3547	0.46±0.08	0.41±0.03	0.37±0.03	0.505
Movement Bouts									
	Number	4149±318	3244±210	2719±145	0.0006; +, *	4183±435	3526±124	3902±259	0.2965
	Duration, s	1.2±0.03	1.18±0.02	1.19±0.03	0.8492	1.14±0.01	1.13±0.02	1.12±0.03	0.8446
	Size, cm	11.89±0.39	11.24±0.24	10.48±0.24	0.0076; *	12.35±0.47	12±0.4	11.56±0.46	0.4592
	Speed, cm/s	10.63±0.48	9.86±0.21	8.98±0.26	0.005; *	11.1±0.41	10.85±0.34	10.53±0.36	0.5587
Feeding Bouts									
	Number	64.17±5.03	56.35±6.94	47±3.02	0.0894	49.02±5.13	48.07±5.2	53.16±4.08	0.7262
	Duration, s	66.51±7.16	87.81±8.51	78.68±4.38	0.1127	73.34±5.46	92.59±8.14	74.66±4.48	0.0671
	Size, mg	63.34±5.16	77.55±6.88	88.12±5.13	0.0206	78.25±7.17	87.88±10.22	75.46±7.3	0.5489

Supplemental Table 1. Measures taken during days 3-7 vs days 13-17 in naive, sham or CCI Balb/c animals. Averages±S.E.M. Significant post-test differences indicated by: naïve vs. CCI: *; naïve vs. SNI: +.

Supplemental Table 2

		SNI Balb/c			SNI Bl/6			CFA Balb/c	
		Naïve	Sham	SNI	Naïve	Sham	SNI	Saline	CFA
Daily Avgs									
	24 hr. Food Intake, g	3.77±0.08	3.92±0.11	3.84±0.06	3.8±0.06	3.81±0.12	3.86±0.07	3.83±0.08	3.85±0.07
	24 hr. Water Intake, g	3.38±0.13	3.31±0.08	3.3±0.09	3.37±0.15	3.29±0.14	3.42±0.14	3.14±0.07	3.25±0.08
	24 hr. Distance, m	714±54	681±36	683±48	277±21	300±25	273±21	381±17	379±12
	DC distance, m	643±43	627±35	626±46	200±15	234±21	217±20	336±14	332±9
	LC distance, m	70.7±15.7	53.7±10.6	57.6±8.7	77.5±8.1	66.6±5.7	56.2±5.8	45.8±7.3	46.9±6.1
Time Budgets									
	Inactive, %	60±1	59.9±0.9	61.9±1	70.7±1	72.9±1.1	71±1.2	61.9±0.8	60.8±0.7
	Stopped, %	28.7±0.7	28.2±0.6	27.42±0.69	20.3±0.9	18.4±0.8	19.53±0.72	23.8±0.6	25.2±0.7
	Locomotion, %	5.85±0.37	5.39±0.24	5.3±0.3	2.86±0.2	3.08±0.23	3±0.2	3.7±0.16	3.77±0.15
	Feeding, %	4.8±0.4	6.1±0.6	4.9±0.4	5.5±0.3	5.1±0.3	5.9±0.6	10.3±0.7	9.9±0.5
	Drinking, %	0.6±0.14	0.43±0.02	0.51±0.06	0.59±0.03	0.52±0.02	0.53±0.03	0.35±0.01	0.36±0.02
Movement Bouts									
	Number	4095±229	3924±148	3613±202	1502±131	1558±141	1443±104	-	-
	Duration, s	1.14±0.02	1.1±0.02	1.16±0.02	1.57±0.08	1.62±0.1	1.72±0.1	-	-
	Size, cm	12.5±0.43	12.45±0.47	13.46±0.61	12.42±0.57	13.48±0.45	12.95±0.24	-	-
	Speed, cm/s	11.05±0.35	11.54±0.52	11.83±0.57	8.64±0.35	9.05±0.32	8.55±0.38	-	-
Feeding Bouts									
	Number	42.59±3.38	65.9±9.43	51.21±3.69	59.09±4.57	59.33±4.04	55.4±4.98	-	-
	Duration, s	81.76±2.66	72.01±4.22	69.96±4.94	75.08±4.6	70.09±7.2	90.92±15.4	-	-
	Size, mg	87.13±6.37	64.91±6.28	74.53±5.8	66.03±4.53	63.77±4.52	73.64±6.13	-	-
	Intensity, mg/s	1.1±0.09	0.93±0.07	1.11±0.06	0.93±0.04	0.99±0.07	1.01±0.07	-	-

Supplemental Table 2. Home cage behavior averages on days 6-17 in animals with SNI and CFA. There were no significant differences using one-way ANOVA on any measure.

Chapter 3

Assessing disability in mice with models of knee osteoarthritis and back pain: A pilot study of two joint degeneration models

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Abstract

While neuropathic pain is often the major focus of pre-clinical animal models, it is relatively rare in the human population. Much more common is joint pain, particularly in the weight-bearing joints: the back, hips, and knees. Here, we addressed knee and back pain in mice. Osteoarthritis of the knee was modeled using a destabilization surgery, where the medial meniscus is displaced. To model back pain, particularly pain related to disc degeneration, we induced degeneration in mice using a stab injury. These two models were behaviorally assessed in three of tests of disability: climbing, rotarod and novel cage exploration. Though clearly leading to physical injury, back pain did not produce any behavioral measures for the 7 weeks of monitoring. Likewise, knee joint destabilization did not produce measurable changes in the tests used even at 14 weeks past the initial injury. While this does not rule out the possibility that these models lead to some pain

experienced by the animals, these models do not replicate the disability caused by pain in many humans with joint pain.

Introduction

Joint pain is the most common type of chronic pain reported in humans. This is particularly true in older populations; of those over 65 years old, 35% of men and nearly 60% of women suffer from osteoarthritis (Perruccio et al., 2006; Wieland et al., 2005). Moreover, the two most common locations of reported pain are the knee (15% of pain reports) and the back (42%) according to a survey of people in 15 European countries. This study also found that 50% of those reporting pain, stated the cause as either osteoarthritis or degenerating discs (Breivik et al., 2006). Though it is clear that the degeneration of the joint in osteoarthritis and disc disease are at least partially responsible for the pain, the mechanisms behind the degeneration and subsequent pain are less well understood.

Given the need for more understanding of the mechanisms involved in joint degeneration-related pain, there are few pre-clinical models of knee osteoarthritis that replicate the degeneration of the joint, rather than simply the accompanying inflammation (Ameye et al., 2006; Bendele, 2001; Neugebauer et al., 2007). The two most common models are monoiodoacetate (MIA) injection and surgical destabilization. Though both lead to degeneration similar to the human pathology, the time course of the two are very different. MIA develops within a few days (Bove et al., 2003; Combe et al., 2004; Ferreira-Gomes et al., 2008), while some of the

surgical manipulations do not show pain and degeneration until many weeks after. In one surgical method, the ligaments that hold the medial meniscus in place are cut, leading to degeneration at 8 weeks post-surgery and a change in locomotion and weight bearing by 10 weeks after injury (Glasson et al., 2004; Inglis et al., 2008; Ma et al., 2007).

While osteoarthritis in the knee has been studied using animals, functional studies of back pain models are almost entirely absent from the pre-clinical literature. More over, though anatomical and physical changes to the discs and surrounding tissue following degeneration have been investigated, behavioral observations in these studies are rare. The most commonly used method to induce degeneration is a stab injury of the disc, damaging the nucleus pulposus (Elliott et al., 2008; Sobajima et al., 2005). Studied in rabbits and rat, there are only two instances where behavior was included as an outcome (Olmarker, 2008; Rousseau et al., 2007). Stab injury has not been used in mice, but recently, a mouse lacking a gene involved in disc structure maintenance, which spontaneously develops degeneration of the intervertebral discs and has some behavioral pain phenotypes, has been investigated. (Millecamps et al., 2010).

The question of what behavior to use as a surrogate measure of pain in models of joint degeneration is problematic. Unlike neuropathic and inflammatory pain models where researchers can use hypersensitivity as at least an approximate tool to characterize the model, it is unlikely that hypersensitivity plays a large role in OA

or disc-related back pain in humans (with the exception of radicular lower back pain.) Nevertheless, researchers have shown that some of these models cause mechanical hypersensitivity. Though the change in threshold may have as much to do with the degeneration induction methods as the degeneration itself. A more reliable method of pain assessment, with a greater face and construct validity, would be the use of disability measures given that humans often show decreased mobility and use of the affected joint (Dieppe et al., 2005; Kidd, 2006). Many of these measures have been used in rats with MIA and joint destabilization, including weight distribution away from the ipsilateral limb (Bove et al., 2003; Combe et al., 2004; Fernihough et al., 2004), gait changes (Ferreira-Gomes et al., 2008), home cage activity (climbing and exploration) (Inglis et al., 2008), and alterations to sleep (Silva et al., 2008).

Here we have characterized models of OA and back pain using behavioral measures of disability. Specifically, we used a disc-stab (DSD) model of interverterbral disc degeneration and medial meniscus destabilization induced osteoarthritis (OA). Mice were then assayed in tests of horizontal and vertical movement, as well as motor function each week for up to 17 weeks.

Methods

Animals

Adult male mice (Balb/c and C57Bl/6) were purchased from Charles River, and arrived at least 2 weeks before testing began. All mice were housed in cages with

corncob bedding and a cotton nestlet in groups of 3 to 5. All cages were changed every two weeks, at least two days before the next behavioral test. Animals had freely available food and water under a standard 12-hour light/dark cycle with a regulated ambient temperature of 20-22°C. Experimental manipulation occurred at 7-10 weeks of age. All procedures were approved by the Institutional Animal Care and Use Committee at UCSF and the guidelines of the Committee for Research and Ethical Issues of IASP. For all experiments, animals were habituated to handling prior to testing.

Experimental models of pain

Disc degeneration (DSD) was induced in C57Bl/6 mice (n= 4-5 per group) by a stab injury to the L4/L5 and L5/L6 lumbar discs similar to procedures in the rat (Rousseau et al., 2007). Briefly, under isoflurane anesthesia, a midline abdominal incision was made to open the peritoneal cavity. Intestines were moved to the side and, after careful dissection of the inferior vena cava, the lumbar discs were exposed. For each of the two discs, a 30-gauge needle was used to make two stab penetrations into the exposed part of the disc. The intestines were returned to their normal position and the peritoneal cavity closed separately from the skin incision with silk suture. In sham animals, the discs were exposed, but not punctured. Post-operative buprenorphine (0.1 mg/kg, s.c.) and bupivacaine (8mg/kg, locally administered) were given to experimental and sham animals. The naïve group receive anesthesia only.

Joint destabilization was induced in Balb/c males (n=4) by dislocating the medial meniscus of the knee, using methods similar to those previously described (Glasson et al., 2007). Here, we made a midline incision over the ventral aspect of the knee, then the medial compartment of the knee was entered via a medial parapatellar approach, without displacing the patella or cutting the quadriceps muscle. After the medial meniscus was identified, its anterior horn was released by dissection of the ligaments attaching it to the tibial plateau. Mobility of the anterior half of the medial meniscus was confirmed by displacement with forceps. In the full sham animals, the meniscus was exposed, but not dislocated. In partial sham animals, the skin was opened only. The parapatellar window was closed with silk suture and the overlying skin closed with wound clips. Post-operative buprenorphine (0.1 mg/kg, s.c.) and bupivacaine (8mg/kg, locally administered) were given.

Behavioral Assays of Pain-Related Behavior

In both experiments, baseline activity was assessed on a climbing apparatus, a rotarod test and a novel cage exploration test. In disc degeneration experiments, these behaviors were assessed once a week for 5 weeks after injury. In OA animals, post-injury behavior was only assessed once every two weeks. Only one test was performed each day, during the light cycle. For all tests, animals were brought into the testing room at least half an hour prior to beginning the test. Between tests, the testing apparatuses were sprayed with dilute bleach and wiped dry.

Rotarod was performed on an accelerating rod apparatus (Ugo Basile), where the time spent before falling from an accelerating rod treadmill was used as a measure of motor function. For training, mice were run until all the animals stayed on for more than 200 sec (at least 2 training days). On subsequent testing days, animals were tested three times. The average of these three trials was used to assess the change from baseline rotarod behavior. To assess vertical movement ability, animals were placed into a 40cm high wire-mesh cylinder with a clear plastic top (Deacon et al., 2005). Behavior was recorded by video for 10 minutes and time spent climbing, defined by all four limbs off the floor, was measured. Novel cage exploration was used as a measure of overall activity. Animals were placed in a clean cage and recorded by video for 5 minutes. Distance moved was assessed using a videotracking system (Ethovision, Noldus).

In DSD animals, anxiety and exploratory behavior was assessed at week 4 in the open field (OF) test. This was performed under normal lab lighting (more than 100 lux). Mice were placed in the OF apparatus, which consists of four white chambers measuring 50 × 50 × 38 cm, allowing 4 animals to be tested concurrently. Mice were allowed to freely explore the chamber for 30 minutes. Each chamber was divided into the outer zone (15 cm from the walls) and the center zone. Activity was recorded by video and analyzed using the Ethovision software. Time spent in the center zone was used as the measure of anxiety.

Histological assessment

At 9 weeks following DSD, lumbar discs and adjacent vertebrae were harvested, fixed in 10% formalin for 5 days, decalcified in formic acid for 2 days, dehydrated, and embedded in paraffin. Six-micron sections were cut parallel to the direction of the stab. Sections were stained with safranin-o, fast green, and hematoxylin.

Results

Disc stab injury causes degeneration

When the discs were observed histologically at 9 weeks after injury, there were a number of changes compared to control discs indicative of degeneration. While each control disc had a rounded, large nucleus pulposus (NP) containing normal patterns of interior staining (green), the NP from stabbed discs were mis-shaped and did not present any staining within the interior (where nuclear cells reside). Additionally, the lamellae of the annulus were no longer as well organized (Figure 1). In those sham and naïve animals that were observed histologically, there was no evidence in any lumbar disc of spontaneous degeneration.

Pain-related behaviors in animals with joint injury

Naïve, sham and DSD animals were observed weekly for changes to climbing, rotarod, and novel cage exploration behaviors. Though behavior in all groups varied over the course of the experiment, potentially due to learning the apparatus, at no point were there any significant differences among the groups. As with DSD animals, those with OA also displayed no differences from either full or partial sham

groups. However, in the case of climbing behavior, the amount of time spent climbing in animals from this group was highly variable, so no conclusions could be drawn.

At week 4 after injury, animals with DSD were observed in the open field test of anxiety and activity. Unsurprisingly, given the results from the novel cage exploration tests, DSD animals did not move any less in the 30 minute test than animals in the control groups. Further, all groups showed similar distance and time spent in the center area of the open field, indicating little difference in anxiety at 4 weeks after manipulation.

Discussion

In this pilot study, animals with models of degenerative joint disease did not display any potentially pain-related behaviors at any time after injury. While these injury models have been shown here and elsewhere to reliably cause degeneration similar to human conditions, they do not cause a similar disability in mice. These results are consistent with many of the findings from our lab, showing that behavior in short-duration tests are unchanged in mice with models of chronic pain. However, other studies of a similar OA model and in genetically prone to disc degeneration have observed that some of these behaviors were altered by the degeneration.

The same medial meniscus destabilization used here, has been shown to have a dramatic change in weight bearing in mice (Bove et al., 2003; Mcnamee et al., 2010).

We did not test this parameter, but gross observation of the mice did not reveal any signs of contralateral preference. Moreover, in the same study, mice also displayed reduced climbing, time spent mobile and distance moved by 8 weeks after injury (Inglis et al., 2008). While we cannot account for the discrepancy, our surgical methods may have been different or our numbers too low. However, a recent study from another group, like our results, did not see locomotor differences at 8 weeks post-injury (Malfait et al., 2010). This same study showed that the prevalence of degeneration is mouse strain dependent and here, we used a different strain (Balb/c) than the original studies (C57Bl/6).

The results from our disc-degeneration model are more similar to the results of the recently published SPARC-null mouse model of back pain (Millecamps et al., 2010). In the study, they did not observe changes to rotarod performance and nor in other locomotor measures (Tajerian et al., 2010). Interestingly, the only disability behaviors they found that changed were in the response to tail suspension and a measure of grip strength. It is therefore unsurprising that we too did not see any behavioral changes with disc degeneration.

There are two major limitations limitation to studying back and knee pain in mice. The first is that comparing the impact of joint issues between a bipedal and quadrapedal animals is not straight-forward. Both the back and knee in humans bear much of a human's weight. In a mouse, the weight is distributed differently between the limbs, which might alter the impact of an injury in the knee or lumbar

region. Secondly, in humans, degeneration of the discs occurs relatively frequently, but severe back pain as a result is less common. A similar phenomenon exists in OA as well, where people with equal levels of degeneration reporting different amounts of pain, suggesting that degeneration alone may not fully model the pain experienced by those with osteoarthritis (Dieppe et al., 2005; Ferreira-Gomes et al., 2008). For the sake of animal models of degeneration however, it is common to assume that any injury will lead to a pain phenotype. Here, we have shown that the disc stab induced degeneration, though sufficient to induce a histological change did not alter the motor ability and motivation of a mouse. Degeneration of the knee joint was also unable to induce major disabilities in the mice. Our results suggest that the use of these models may be valuable if other outcome measures can be found, but that the measures most similar to human pain-related disability were not altered.

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Figure 1

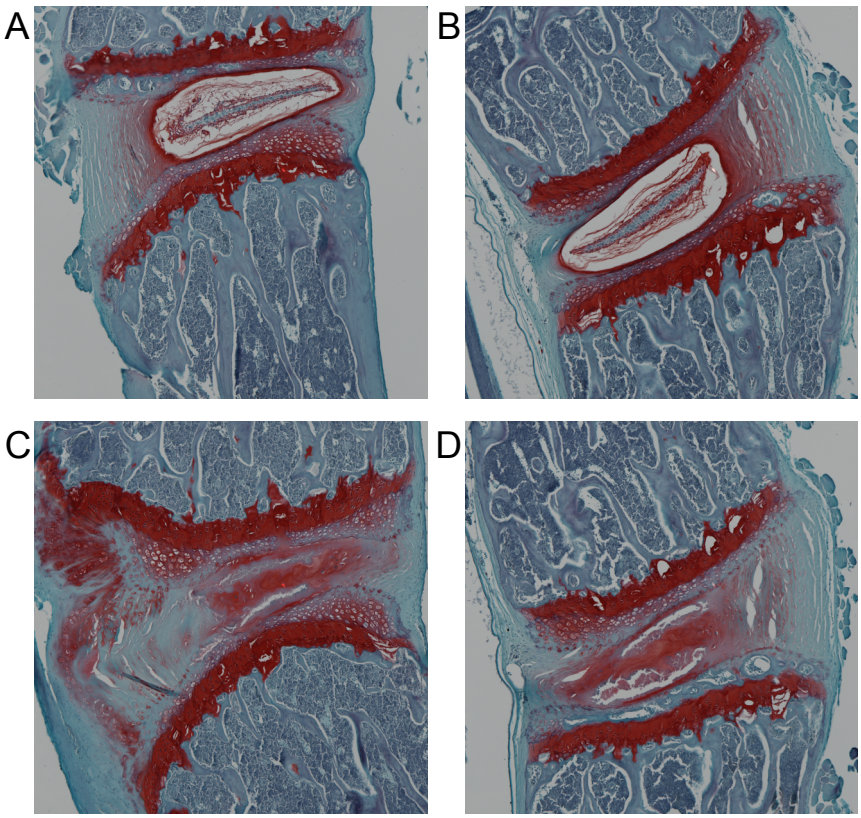

Figure 1. Histological analysis of intervertebral disc with and without disc stab injury. (A-B) Control discs from two animals showed normal joint structure and well formed discs. (C-D) At 9 weeks after stab injury, injured discs in the same two animals as in A and B no longer had rounded, distinct nucleus pulposus and the lamellae of the annulus are bulging and less organized.

Figure 2

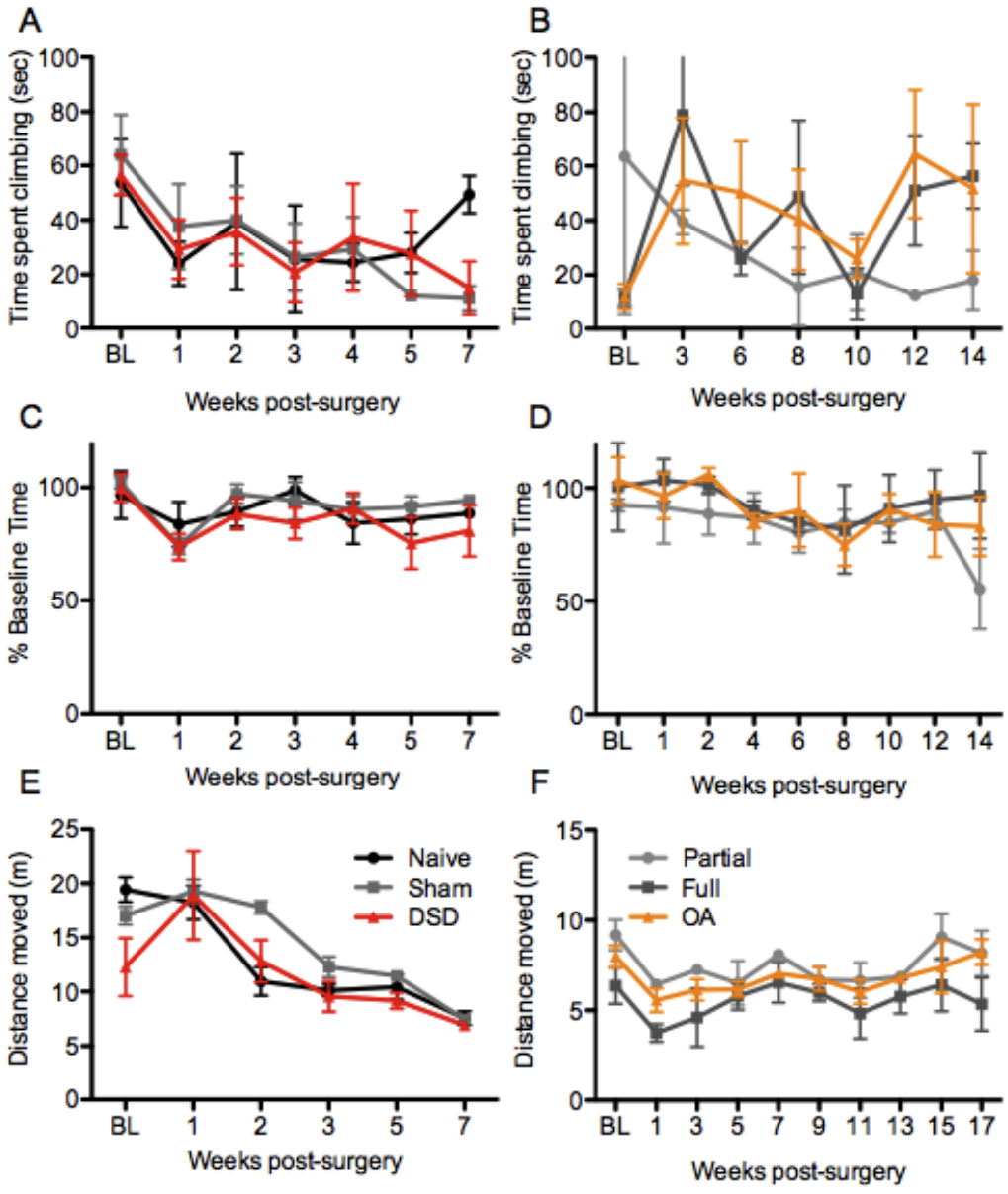

Figure 2. Animals with (A,C,E) DSD and (B,D,F) OA did not have altered behavior in (A,B) the time spent climbing on a wire mesh during a 10-minute period, (C,D) time spent on the accelerating rotarod compared to baseline performance and (E-F) the distance moved during a 5-minute exploration of a new cage.

Figure 3

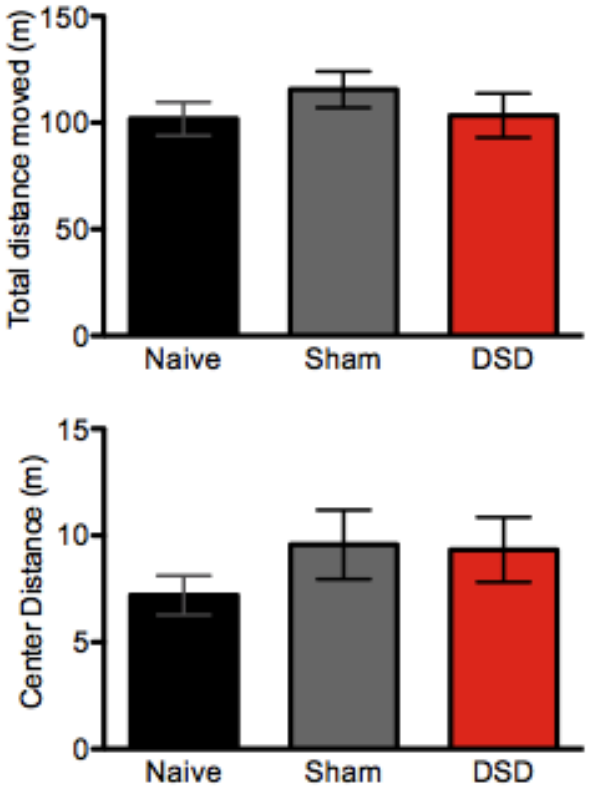

Figure 3. Open field behavior in animals with disc stab injury. (A) Animals did not move any less in the 30 minute open field test compared to controls. (B) DSD animals explored the center area of the field as much as controls, indicating that they all have similar state anxiety levels.

Chapter 4

Dental pulp injury has a short-term effect on consumption of sucrose and weight gain, but no effect on normal feeding in mice

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Abstract

Pain from an inflamed dental pulp, often due to exposure, is a common cause of orofacial pain. The pain can be severe and persistent, often requiring an irreversible dental procedure, such as an extraction or root canal, to alleviate the pain. It is thus essential to develop an animal model of pulpitis to aid in understanding the mechanisms of dental pain and to find methods for assessing that pain. Here we used a mouse model of pulp exposure (namely dental pulp injury; DPI), in which an early period of inflammation is followed by pulpal necrosis, induced by exposing the pulp of the maxillary first molar. Following injury, we monitored the daily life behaviors of mice with DPI, including measures of eating, drinking, and movement. While both anesthesia controls and DPI animals decreased feeding and overall activity in the first few days after manipulation, there was no additional effect of dental injury on any parameter except locomotion in the first 48 hours. Despite the lack of change in feeding, there was a significant decrease in the amount of weight gained by DPI compared to control animals following manipulation. To find a simple assessment of dental pain, we also used a test of sucrose consumption. In this

assay, mice were given access to 2.5% sucrose at the start of the dark cycle and the amount of liquid consumed was measured. Before injury, all mice consistently drank a similar amount of sucrose on each baseline test day. Mice with bilateral DPI increased their consumption of 2.5% sucrose to over 150% of baseline consumption. This increase lasted for the first week after DPI. The increase in both weight loss and sucrose intake in the first 2 days of injury is reversed by administration of indomethacin, but not naloxone. Our findings indicate that sucrose consumption is a reliable measure of dental pain, but the underlying cause suggests complex alterations on energy consumption and motivation of an animal with pulpal inflammation.

Introduction

Dental pain is a common problem in the clinic, affecting an estimated 12-14% of adults in the U.S. annually (Lipton et al., 1993). This is particularly the case in populations that do not have access to sufficient preventative care, in one study over 40% of low-income adults experienced 5 or more episodes of toothache in the preceding 10 years (Cohen et al., 2009). Epidemiology of dental pain in adults, and particularly children, show that much like other chronic pain conditions, dental pain leads to changes in quality of life. Researchers have addressed how toothache influences sleeping, eating, socializing, mood and self-image, in addition to the pain itself (Allen, 2003; Cohen et al., 2009; Marbach et al., 1981; Reisine, 1988; Sanders et al., 2009; USDHHS, 2000; White et al., 2003).

Given the importance of understanding the underlying mechanisms of dental pain and its subsequent alteration of a patient's quality of life, there are few rodent behavioral models. Therefore, it is essential to develop an animal model of pulpal injury to aid in understanding the mechanisms of dental pain and assessing analgesic strategies. There are a number of pulpitis models in the rat, many of which include pulp exposure (Byers et al., 2000; Chudler et al., 2005; Khan et al., 2008; Locher-Claus et al., 2005; Tarsa et al., 2010). These exposures are most often in the molars of rodents, where the distribution of pulpal fibers is similar to in a human tooth (Byers et al., 1999). Some pulpitis models go further by introducing lipopolysaccharide (Chattipakorn et al., 2002; Okiji et al., 1991), or other inflammation-inducing agents (Chidiac et al., 2002; Kawamura et al., 2010; Sunakawa et al., 1999). These exposure models have mainly been studied in terms of anatomical and physiological changes in the tooth, trigeminal nucleus and the caudalis. However, while many mechanisms have been elucidated anatomically, it is still unclear to what extent these specifically relate to dental pain. The primary goal of the present study is to demonstrate the utility of novel behavioral outcomes to measure dental pain in mice.

Finding reliable surrogate measures of pain in mice has been problematic in many chronic pain models, from knee arthritis to back pain to neuropathy (Blackburn-Munro, 2004; Mogil, 2009). Unlike many of these models, which were originally characterized using evoked measures of hypersensitivity after injury, dental pain as yet does not have a standard method for behavioral assessment. Indeed, there is a

dearth of even a simple evoked hypersensitivity dependent measure of "pain" in animal models of pain. Though in rats, mechanical testing of the facial skin has been measured (Grelik et al., 2005; Ono et al., 2009), this has not been possible mice.

To measure pain behaviors associated with dental and other orofacial pain conditions, such as tempromandibular joint (TMJ) pain, researchers have used a number of different outcomes including feeding (Ansah et al., 2010; Kramer et al., 2010), biting (Khan et al., 2008), jaw-opening (Takeda et al., 2009) and sleep (Schütz et al., 2003, 2004). Some have shown that dental pain models, including pulp exposure, in rats and mice causes animals to groom the face more (Chattipakorn et al., 2002; Chidiac et al., 2002; Yang et al., 2009) and reduce activity in an open field (Chudler et al., 2005; Shibazaki et al., 2009). More direct measures of biting and eating activity have also been used. The Dolognawmeter, for example, has been recently developed to measure the length of time it takes an animal to gnaw through a plastic bar and reliably indicated pain-related disability due to TMJ inflammation and oral cancer (Dolan et al., 2010). Similarly, meal patterning, i.e. the temporal architecture of feeding, was shown to be a reliable outcome for TMJ-related pain, as well as pulp exposure, finding that rats with orofacial pain increased the time spent for each meal, without a large effect on the total food consumed (Bellinger et al., 2009; Harper et al., 2000; Kerins et al., 2005; Kramer et al., 2010). Lastly, operant models that stimulate the orofacial region have been used to measure the thermal sensitivity of animals, though this method has not yet been put to use in the setting of injury (Neubert et al., 2008; Rossi et al., 2006).

While these behavioral measures have begun to allow for the understanding of orofacial pain in rats, there are currently far fewer measures in mice, despite the species' utility in and ease of genetic manipulations. Thus, the goal of this study was to evaluate a model of pulp exposure in mice in terms of possible behavioral measures of dental pain, particularly those that might reflect more than simply evoked sensitivity. First, we measured the daily life of mice with dental pulp injury by monitoring their home cage behaviors, including assessing feeding, drinking and movement. Secondly, we characterized and validated a modified operant task of sucrose consumption as an indirect measure of dental pain.

Methods

Animals

Adult male and female C57Bl/6 mice were purchased from Charles River Laboratory, and arrived at least 2 weeks before testing began. All mice were housed in cages with corncob bedding and a cotton nestlet in groups of 3 to 5, unless separated due to fighting issues, which occurred occasionally in male mice. All cages were changed every two weeks, at least 24 hours before the next behavioral test. Animals had freely available food (Purina 5058 chow, pellets) and water under a standard 12-hour light/dark cycle with a regulated ambient temperature of 20-22°C. Experimental manipulation occurred at 10-12 weeks of age. All procedures were approved by the Institutional Animal Care and Use Committee at UCSF and the guidelines of the Committee for Research and Ethical Issues of IASP.

Experimental dental pulp injury (DPI)

We created a dental pulp injury (DPI) in mice by mechanically exposing the tissue of the pulp, which has been shown to produce pulpal inflammation (pulpitis) followed by necrosis of the pulpal tissues (Supplemental Fig. 1). Procedures were performed under ketamine-xylazine anesthesia (100-10mg/kg, respectively). For DPI, animals' mouths were propped open using curved forceps. Then the maxillary first molar was drilled with a ¼ round bur at low speed until two pulp horns were exposed. This was done either unilaterally or bilaterally. Drilled area was cleaned with canned air and saline, and then left exposed. Control animals received anesthesia and also had their mouths propped open for a similar length of time as the DPI animals (approximately 10 min). After initial recovery from anesthesia, animals were returned to their cages, where they had access to water-softened food pellets.

Home cage monitoring (HCM)

To monitor behavior in the home cage of mice with dental injury, we used the automated monitoring system developed by Goulding, et al (Goulding et al., 2008). Briefly, this system consists of 32 cages (with betachip bedding, plastic niche and cotton nestlet) each placed on a pivoting platform with two load beams calibrated to detect position of the mouse, with photobeams at the feeder (containing Purina 5058, powdered food) and a lickometer at the water bottle to detect bouts of feeding and drinking. Daily monitoring proceeded continuously, except for 1.5-2 hours daily maintenance on the system, when the previous day's food and water were removed

and weighed before replacing with new food and water. This maintenance always occurred between 10:00 and 16:00.

The 32 cages were evenly divided between DPI and control groups. All animals were initially put in the system for 9 days to measure baseline activity (Fig. 1A). On the day of DPI/sham manipulation, animals were removed, weighed and immediately given anesthesia. As soon as they recovered from anesthesia, animals were returned to their home cage in HCM system. Monitoring continued when all animals had been returned (system was off for a total of 5 hours). Using methods developed for the system, data were checked for errors and activity classified as inactive or active. Within the active state, mouse behavior was further classified based on location, movement, and feeder/lick spout data as feeding, drinking, moving, or other (which includes small movements and can be separated by location.) Note that only half the animals in each group had enough days of complete data to be included in the detailed analysis of bouts and time budgeting, mainly due to data lost by photobeam blocks by powdered chow at the feeder.

Sucrose consumption assay

All sucrose consumption assays were performed beginning 40 minutes before the start of the light cycle. To perform this test, food and water were removed 3.5 hours before the start of the test to reduce variability in the timing of each mouse's last meal. At that time, body weight was measured and cages were brought into the testing room. Sucrose solutions were made fresh for each test and put in bottles

filled with reusable ice cubes (Icy Cools®) that were either frozen, when testing cold sucrose consumption, or thawed, when testing room temperature consumption. The bottles were weighed just prior to the start of the test. The test began when each animal was introduced to a separate test cage, containing one sucrose bottle. The animals were left to drink freely for 2 hours, after which they were removed from the test chambers and returned to their home cages. Sucrose bottles were weighed and, in the case of cold sucrose tests, the temperature of the solution was measured (if the temperature was above 6°C that animal's data were excluded.) Each test day was at least 48 hours (and no more than 4 days) after the previous test. Note that in the tests of cold sucrose consumption for mice with only unilateral DPI, a second bottle containing RT water was also available during the test. However, because animals rarely drank from this bottle (0.3±0.01g), we do not report the results here.

To train animals in the room temperature (RT) sucrose consumption assay, we first exposed them to 2 days of 5% room temperature sucrose. Animals were then trained at 2.5% for 4 additional days, at which point all animals reached a stable baseline. We then performed 3 tests to assess baseline sucrose consumption. For the cold assay, animals received RT sucrose on the first two training sessions (once at 5%, once at 2.5%). Then animals received the same training and baseline schedule as the RT animals, except with cold sucrose. Any animals that failed to have an average baseline consumption of 0.45g were excluded from the study, which rarely occurred in animals consuming RT and in about 10-15% of those

consuming cold sucrose. The morning after the 3rd baseline, animals were assigned to control or DPI groups, in a manner allowing even distribution of baseline sucrose consumed and cages (to prevent any cage from having all control or all injured animals.) Animals were all drilled between 9:00 AM and 1:00 PM. Animals were then tested on days 2, 5, 7, 9 (or 10), 12, 14, 17 and 19 (or 20) after DPI or sham manipulation (See Figure 1B for timeline of room temp tests.) In these experiments the outcomes measured were both total sucrose consumption scores (in grams of liquid consumed) and percent change from the average baseline consumption.

Drug studies

To test the sensitivity of the DPI-induced sucrose behavior to an NSAID, we gave mice 5-6mg/kg indomethacin (Sigma, in 1.7% ethanol and 0.9% saline) per day for the first 4 days after injury. Each injection (subcutaneous) was given at the start of the dark cycle, except on day 2 after DPI when the injection was given immediately following sucrose consumption testing (1.5 hours after lights out). Thus, at the time of sucrose testing on days 2 and 5, drug had been given nearly 24 hours prior. To habituate animals to any effects of the injections themselves, animals were given an injection of saline 24 hours prior to each sucrose test during the training and baseline periods. One animal was excluded because the injections of indomethacin were unsuccessful on two consecutive nights (animal moved during injection and most liquid leaked out.) To test the involvement of endogenous opioids in sucrose consumption behavior, we adminstered 1mg/kg naloxone (i.p.) 30 minutes prior to sucrose testing. We tested once immediately after training (before injury) and on

day 2 after DPI or sham manipulation. To control for possible effects of the injection, during all other training and baseline test days, animals received a saline injection prior to the test. (Fig. 1B)

Statistical Analysis

Results are expressed as mean±SEM and *p* values less than 0.05 were considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Comparisons were analyzed with one-way or repeated measure analysis of variance (ANOVA). In experiments with only one control group, we used Student's t-test, except in cases where data were non-parametric, for which the Mann-Whitney u-test was used. For ANOVAs, post-hoc analysis used Bonferroni tests. Data were analyzed using GraphPad Prism 5 for Mac.

Results

Weight changes after DPI

All animals lost weight as a result of the anesthesia and/or sham or surgical intervention. Control animals, which only received anesthesia and had their mouths propped open, lost about 2% of their body weight as measured on day 2 compared to the day the manipulation was made. During this same period, animals in the bilateral DPI group lost twice as much weight (control, -2.3±0.7; DPI, -4.4±0.4; $p<0.05$, Fig. 2A.) Interestingly, this greater amount of weight loss was not due to simply more weight lost on the day of anesthesia, as there was no difference if measured at 6 hours after injury (Control -4.0% vs. DPI -4.1% weight change, n=15-

17). Indeed it is clear when looking at the weight change from day 1 to 2 after injury, where control animals already returned to normal daily weight gain, DPI animals were still losing weight (control, 0.28±0.14g vs. DPI, -0.05±0.08g). It is only by day 5 that they fully recovered to normal daily weight gain (two-way ANOVA: effect of treatment $p=0.001$, $F= 12.01$, post-test $p<0.01$ on day 2 after injury, n=28-30, Fig. 2B). The pattern of weight loss in animals with dental pulp injury also occurred in male mice with only a unilateral injury and female mice with bilateral DPI (Fig. 2A.)

Daily home cage behavior in animals with DPI

To better observe the behavior responsible for the altered weight gain in DPI animals, we monitored the home cage behavior of animals before and after the sham or DPI procedure. The home cage monitoring (HCM) system used houses one mouse per cage and allows for continuous monitoring of the movements and activities of the animal. All animals were initially monitored for 9 days to habituate them and obtain baseline measures of eating, drinking and movement. At baseline both groups of animals had similar total daily life measures. In the first 9 days, animals of both groups ate 31±0.5g of powdered chow total. Control animals drank 28±0.8g of water and moved 8.7±0.7km total in the 9 days before manipulation. DPI animals had very similar measures, drinking 31±1.7g and moving 8.9±1.2km. (Fig. 3 A-D)

In the 12 days after manipulation, the total movement was similar amongst the two groups and monitoring periods (post manipulation: control, 10.0±1.2km; DPI, 9.5±1.7km), while intake totals of both food and water increased compared to baseline (feeding: 41±0.8g in both control and DPI, baseline vs. post-manipulation, $p<0.001$; drinking: control, 38±0.8g and DPI, 44±3.6g, baseline vs. post-manipulation, $p<0.01$). Despite the similarity in total food intake between sham and DPI groups, DPI animals failed to gain weight normally. Though the two groups gained similar amounts of weight in the baseline period (5.6±0.9% weight gain in controls vs. 6.2±0.7% in DPI animals), in the 12 days after manipulation, the total amount of weight the two groups gained was significantly different. Control animals gained the same percent weight as they did in the baseline period (control, post-manipulation: 6.0±1.1%), but DPI animals only gained 1.8±0.9% during the twelve monitoring days (One-way ANOVA, $F=4.55$, $p=0.0062$; post-tests $p<0.01$ Pre vs. Post DPI and Post DPI vs. Post control). This difference in weight gain is particularly intriguing, given the lack of difference in total food consumed.

Though feeding, drinking and movement totals for the two groups were not different; we analyzed the data on each experimental day to observe any potential difference in intake or movement for the initial days after injury. When compared to average daily baseline intake/movement, there was a large decrease in feeding, drinking and movement in all animals, regardless of experimental group in the days after anesthesia and manipulation. In the first 24 hours after DPI or sham procedure, movement was 44±5% of baseline in control animals and only 28±3% in

DPI animals (p=0.0101 between the two groups, Fig. 3E) and neither group fully recovered back to 100% until a week later. Likewise, feeding decreased in both DPI and control groups to 80% of baseline amounts (control, 81.3±3.2%; DPI, 80.7±3.3%), recovering within a week after, but without differences between groups on any post-injury day (Fig. 3F). Though water intake decreased by 15% in the first 24 hours after manipulation, intake returned to normal by 48 hours after anesthesia, with no differences between DPI and controls (Fig 3G).

Given the dramatic decrease in movement and the intake of food after anesthesia, we analyzed the circadian patterns of the first two post-manipulation days. Baseline behavior in the home cage peaked in the early hours of the dark period, when measured either by distance moved or hourly food intake, with waning activity as the night progressed (Fig. 3H-I). This peak of activity did not occur during the first night after anesthesia, which was between 3.5-5 hours after initial recovery (i.e. ambulation). The circadian pattern of both eating and movement returned to the normal early nighttime peak by the second night (approximately 28 hrs after initial recovery from anesthesia.)

In the rat, it has been shown that the architecture of feeding was altered with pulpal injury, without much disruption of total food intake (Bellinger et al., 2009). Therefore, we analyzed the eating and movement patterns of animals in the days after injury. The HCM system allows for the detailed analysis of time budgets and movement and intake bouts. Though the first day after anesthesia/manipulation

had an effect of the intake and movement bout properties, there were no differences between controls and DPI group in any measure (Table 1). In both groups of mice, for the first 24 hours, feeding bout number decreased, while the size and duration of each bout increased.

Sucrose consumption in non-manipulated mice

The home cage behavior did not produce a measurable outcome of dental pain in mice with pulpal exposure. To develop a reliable behavioral assay of pain in these animals, we used a modified sucrose consumption test. Naïve male mice drank significantly more room temperature (RT) 5% sucrose than RT 2.5% sucrose (2.30±0.08g vs. 1.14±0.08g, $p<0.001$), and more RT 2.5% than Cold 2.5% (1.14±0.08g vs. 0.72±0.08g, $p<0.001$; Fig. 4F). Most animals drank more sucrose of either temperature or concentration than RT water (0.33±0.02g), though there was an occasional animal that did not drink any cold sucrose. These animals were not included in the DPI studies. This indicates while mice preferred RT to cold liquids, they still were motivated to drink sucrose regardless of temperature. In fact, when animals were given a direct choice of drinking 5% RT or cold sucrose, mice drank 6 fold more RT than cold (0.92g vs. 0.15g, $p<0.001$). Even when given a direct choice between 2% RT vs. 5% cold, the animals still preferred drinking RT (0.70g vs. 0.33g, $p<0.001$). Nonetheless, once animals were trained on either 2.5% RT or cold sucrose, the amount they drank in each two-hour session remained consistent.

Sucrose consumption after DPI

Animals received training on the consumption test until their baseline consumption was stable, then animals received DPI or sham manipulation. On the second day after DPI or control manipulation (approximately 53 hours after initial recovery from anaesthesia), male mice with bilateral DPI increased the amount of sucrose consumed to over 150% of baseline, while controls remained at baseline consumption levels. Originally we had hypothesized that cold might be come more aversive after injury, and thus that DPI animals would reduce their consumption of cold sucrose. However, there was not an effect of temperature on the percent increase in DPI animals, (RT DPI: 176±14% vs. cold DPI: 207±31%, $p=0.64$). For both temperatures, consumption remained elevated for over a week after manipulation, however only in the room temperature group was it significant beyond day 2 (cold 2-way ANOVA, effect of treatment $p=0.05$, post-hoc t-test at day 2 $p<0.05$; RT 2-way ANOVA, effect of treatment $p=0.005$, post-hoc t-test on days 2-9 $p<0.05$; Fig. 4B&D). Further, the average consumption for the first week after manipulation (normalized to baseline) was significant with both cold and RT in male mice with bilateral DPI (Cold, control 117±10%; cold, DPI 174±16%, t-test $p<0.01$ compared to control; RT, control 120±12; RT, DPI 195±27, t-test $p<0.05$ compared to control; Fig. 4E). Unlike with bilateral injury, male mice with only a unilateral DPI did not have a significant increase in cold sucrose consumption compared to controls (134±11% of baseline vs. 132±13%; Fig. 4E). However, as the unilateral experiments were run with a room temperature bottle of water available

throughout the 2-hour test, it is possible that these two consumption scores are not directly comparable.

DPI-induced increase in sucrose consumption is not dependent on gender

There have been a number of studies in mice showing that pain behaviors can be gender dependent (Mogil, 2009; Painsipp et al., 2007). Therefore, we also ran the sucrose consumption test in female mice with DPI. Baseline consumption behavior was very similar to male mice (ranges of sucrose consumption with both RT and cold: 0.3g to 2.3g in males and 0.3g to 2.6g in females). On day 2 after manipulation, female mice with DPI also increased consumption of cold sucrose by 150%, with an average increase in the first week of 165±10% of baseline compared to 101±9% in controls ($p<0.001$; Fig. 4E). The same increase occurred with RT sucrose in female mice with bilateral DPI (154±13%baseline in DPI animals vs. 119±6% in controls). As there were no differences between genders or temperatures, for the remaining pharmacology experiments, female mice were used with RT sucrose.

Relationship of sucrose intake, weight change and feeding

To better understand what might be contributing to DPI animals' increased sucrose intake, we examined the relationship of weight change to sucrose intake. Before manipulation, using baseline sucrose consumption and daily average weight change there was no correlation between weight and sucrose consumption (Pearson $r = -0.06$, $p=0.27$). When data from all animals (DPI and sham) on day 2 after manipulation was used for analysis, there was a significant inverse relationship

between percent of baseline sucrose intake vs. percent weight change (n=94, Pearson r= -0.478, p<0.0001). However, the correlation of weight loss to percent of baseline intake was not significant when only DPI animals were included in the analysis (r=-0.17, p=0.25, n=48), indicating that it is likely not simply the weight loss that drives the sucrose behavior.

We also observed sucrose consumption behavior after a short period (12 hours) of food and water deprivation. While the deprivation did motivate animals to drink more 2.5% sucrose, it was to a lesser extent than that of DPI animals (control, 94±4% of baseline vs. deprived animals, 120±7%, p=0.006, n=8-10, Supplemental figure 2B), indicating that mild hunger and thirst were not as motivating as the effects of DPI were in the sucrose consumption behavior.

Sucrose consumption and weight change with indomethacin

To test the validity of the use of sucrose consumption as a measure of dental pain, animals were given daily injections of 5mg/kg indomethacin after DPI. In this case, we administered the drug at the start of the dark cycle, to demonstrate that the sucrose consumption was dependent on the pain experienced throughout the day, rather than just during the test itself. The NSAID had no effect on sucrose consumption in sham-manipulated animals (vehicle-injected, 119.1±6.2% baseline; indomethacin, 119.0±3.9%). Female mice with bilateral DPI given only a vehicle injection increased their sucrose consumption, but those given indomethacin did not (vehicle DPI, 153.8±13.4% baseline; indomethacin DPI, 125±5.0%; one-way

ANOVA: F=4.3, p=0.01; Fig 5B). This same pattern of consumption was also apparent on day 5 after manipulation (Supplemental Figure 1.) Indomethacin also prevented the increased weight loss in mice with DPI during the first 2 days after injury (one-way ANOVA: p=0.02, F=3.5; Fig. 5A). The effect of indomethacin suggests that both sucrose intake and weight change might be good measures of inflammatory pain associated with dental injury in mice.

Naloxone does not alter baseline sucrose intake or DPI-induced increased consumption

To try to determine the extent to which the endogenous opioid system plays a role in the increased sucrose intake by animals with DPI, we administered naloxone (1 mg/kg) or saline 30 min prior to the sucrose consumption test. Though at this dose naloxone has been shown to decrease motivation to drink sucrose, in this case, naloxone had no effect on the amount of 2.5% sucrose consumed (Fig. 6A). However, it is also known that naloxone is more effective the more palatable/rewarding the substance, with 2.5% on the lower end of palatability. Since the motivation to drink sucrose after DPI was increased and thus likely to have increased its reward value, we also tested the same 1mg/kg dose of naloxone on day 2 after DPI. Here too, there was no effect of naloxone on sham animals (109±8% baseline) or on DPI animals (DPI-saline, 163±15% baseline; DPI-naloxone, 154±17%; one-way ANOVA, F=4.4, p=0.03; Fig. 6B), indicating that the endogenous opioid system may not be involved in the DPI-induced sucrose consumption.

Discussion

While there is a clear benefit to using pre-clinical models in the study of dental pain, there are currently few reliable behavioral measures of dental pain in rodents and even fewer specifically for mice. Here, we have demonstrated the use of a novel measure, sucrose consumption, to assess dental pain and shown that other measures of daily behavior did not change as a result of DPI. Specifically, mice with DPI ate and drank the same amounts after injury as did control mice. DPI animals did show a greater decrease in locomotion during the first 48 hours after injury compared to controls. Intriguingly, though food consumption was unchanged, DPI animals gained significantly less weight than did controls. Additionally, in the first week after DPI, injured animals, but not controls, increased their intake of sucrose in a 2-hour test. This increase in consumption was not affected by the temperature of the sucrose or by administration of naloxone, but was eliminated by indomethacin. Though these data indicate that both weight loss and sucrose consumption might be a useful tools to measure dental pain, they also raise a number of questions regarding the causes of the behaviors, including why there is a change in weight gain without a change in feeding and what is driving the increase in sucrose consumption.

Weight loss after dental injury

Both in animals observed during the sucrose consumption test and in the home cage monitoring system, a deficiency in weight gain was observed after DPI relative to controls. A similar relationship between dental pain and weight has been found in

children and older adults with dental pain (Miller et al., 1982; Ritchie et al., 2000). The findings in children are particularly compelling as many dental caries go untreated. A review from Shieham (Sheiham, 2006) suggests there are a number of possible causes of the failure to gain weight. One possibility is that it simply hurts to eat and therefore they eat less. That dental pain can affect eating has been shown in many clinical studies of adults, where, in one study, nearly 60% of patients with toothache responded "a lot" to the question of if the pain kept them from eating (Cohen et al., 2009).

While in humans a change in ability to eat seems like a reasonable hypothesis, our data suggest that this is not the case in mice with dental pulp injury. Here we have shown that although all animals, whether injured or not decreased food intake in the first 3 days after anesthesia, there was no additional decrease of feeding due to DPI. Intriguingly, though both groups exhibit these decreases in feeding behavior, only animals with DPI exhibit a lasting change to weight gain. Further, there was no evidence that more complex aspects of feeding behavior, such as feeding bout properties were different in DPI animals compared to controls. That feeding was unaltered due to pain is perhaps unsurprising, given the evidence in rodents that feeding is a protected behavior, unchanged by injection of formalin (Foo et al., 2009) Even in rats with extractions of all the molars, there is little change to weight or food intake (Yamazaki et al., 2008). This also suggests it is the pain/inflammation itself responsible for the change in weight gain in mice with DPI.

An alternative explanation for weight loss in children with severe caries suggested by Sheiham in her review is that the there might be a direct effect of the dental assault on metabolism. Though chronic inflammatory diseases, such as rheumatoid arthritis as well as cancer, are often associated with cachexia-anorexia (Dantzer, 2001; Langhans, 2000; Means et al., 1992; Plata-Salamán, 2000), it is unclear that chronic pain and/or localized inflammation alone are enough to lead to the pro-inflammatory cytokine-mediated decrease in weight. However, there is a study in older adults with chronic non-malignant pain that shows a relationship between pain ratings and appetite measures, but not weight loss specifically (Bosley et al., 2004). Interestingly, if there is a systemic increase in the cytokines like those involved in cancer-related cachexia, such as IL-1β, with dental pulp injury, then mice should also have decreased appetite. Indeed, it has been shown that systemic injections of IL-1β decreased both food intake and the motivation to drink sucrose (Merali et al., 2003). While investigations into metabolic changes in animals with dental pulp injury are ongoing, it would be a surprising finding that a local injury would have such a pronounced systemic effect and yet no effect on food intake.

Lastly, because we have ruled out a change in feeding behavior and because a change in metabolism without a decrease in feeding would be unexpected for such a localized injury, the decreased weight gain in animals with DPI might be due to a mechanical change in mastication behavior. While an effort was made to observe the chewing in mice with DPI, even at close range, the camera did not allow for the precise analysis of the time spent chewing. Furthermore, while animals in the

sucrose study ate standard pelleted chow, those in the home cage monitoring groups were given a diet of powdered food. Observations of these mice indicated that even with the powdered food there was some time spent "chewing" after the animal initially put food into its mouth. Therefore, an effect on chewing efficiency might help explain the change altered weight gain in DPI animals. In human studies that examine the effect of dentition on the chewing efficiency and nutrition, there is evidence that a decrease in chewing efficiency (due to full dentition) was associated with a decrease in protein absorbance (Rémond et al., 2007).

Animals with DPI increase sucrose consumption

Mice with pulpal exposure increased intake of a sweet solution regardless of its temperature. While we have shown that this behavior is both reliable and reversed with analgesic, we have not been able to demonstrate conclusively the motivating force behind the increase. While clearly there is some relation to the effect on weight gain, the correlation between day 2 weight loss and sucrose consumption was not significant when using data from DPI animals, indicating that weight changes alone might not fully explain the data.

Sucrose itself has been shown to dampen pain behavior in infants and rodents. Therefore, the increased motivation to drink sucrose after DPI might be do to the analgesic properties of sucrose itself. Sucrose analgesia, mainly studied in human infants, has been shown to be involved in suppressing secondary affective pain behaviors, such as crying and facial expressions, rather than actually altering the

sensory aspects of pain (Blass et al., 1999; Slater et al., 2010). In rodents, the direct effect of sucrose on baseline thresholds has been shown to only exist in young pups up to 21 days old (Anseloni et al., 2002). There is, however, also evidence that chronic exposure to sucrose can shift the dose response of morphine analgesia (D'Anci et al., 1997; Segato et al., 1997). In both pups and adult animals, the analgesic effect of sucrose and other highly palatable foods has been linked to the endogenous opioid system and is reversible with naloxone (Anseloni et al., 2002; Foo et al., 2005; Segato et al., 1997). In our experiments, a moderate dose of naloxone did not alter the increased motivation to drink sucrose after DPI, suggesting that analgesic properties do not drive the DPI-induced behavior.

While results from the naloxone study suggest that sucrose analgesia was not the primary driver for DPI animals to drink more, there are two limitations this data. First, in this experiment we only administered one dose of naloxone, which may have been too low to see an effect. In CD-1 female mice, 1 mg/kg is enough to reduce the preference of 5% sucrose over water (Agustín-Pavón et al., 2008). Moreover, different strains of mice respond differently to opioid receptor antagonists: sucrose consumption (10%) over a two-hour period in C57Bl/6 animals was decreased with nearly all doses of naltrexone (0.01 to 5 mg/kg) but Balb/c animals barely decreased consumption at even the highest dose (Dym et al., 2007). Both of these examples also point to a second major limitation in our naloxone experiment, which is the relatively low sucrose concentration. The opioid system is preferentially more involved in sucrose intake at the higher end of

palatability (Hayward et al., 2006; Taha et al., 2006), which might mean that at the lower concentration used here, other circuits might be responsible. The dose of naloxone we gave had little effect on baseline naloxone behavior, suggesting that to fully rule out opioid mediated analgesia, we may need a higher range of doses.

If indeed the naloxone results can be taken to show that there was little opioid-mediated analgesic property of the sucrose itself, then there are a few other possible explanations of why DPI causes an increase in sucrose consumption. The indomethacin results suggest that the increased sucrose consumption was related less to the pain experienced specifically during the testing time, but more to the overall pain experienced throughout the previous day. Because the last dose of indomethacin was nearly 24 hours prior to testing, it is unlikely to still be active in the animal, yet it completely reversed the DPI-induced increase in sucrose consumption. That the indomethacin also had an effect on weight suggests there is a relationship between the two measures, despite the unclear correlation of weight to sucrose consumption within the DPI group. However, as discussed above, because the cause of the abnormal weight gain was not due simply to decreased food intake, the motivation to drink was also not due simply a direct change in feeding behavior. However, if there was a metabolism increase without an increase in food intake or if the food they did eat was not as efficiently digested due to alterations in chewing, then the increase in sucrose consumption could be attributable to the increased motivation to consume calories via non-chewed routes.

An interesting follow up study would be to observe the sucrose behavior in DPI mice with a purely liquid diet.

One last limitation of the sucrose intake experiments concerns the results of days 5-9 after DPI. It is unclear whether the sustained increase in sucrose was due to the motivating driver of the behavior continuing throughout those days or because the animals simply learned to drink more and the drop off by day 10 was due to extinction of this learned behavior. We did not directly test this, but we have data that support both possibilities. The fact that DPI animals receiving indomethacin on days 2-4 did not show an increased consumption of sucrose on day 7 suggests that behavior on this day was dependent on prior sucrose and/or pain experiences on earlier post-manipulation days. However, in animals that were food and water deprived for 12 hours, the increased sucrose consumption did not continue past the day of the deprivation, indicating these animals did not learn to consume more sucrose (Supplemental figure 2). Because we cannot conclude for certain from either of these results whether the prolonged sucrose intake after DPI was due simply to learning or not, we have currently restricted our conclusions from the sucrose test to the earliest days after injury.

Conclusion

There are currently few ways to measure dental pain in mice, but here we present a simple model, DPI, and two outcome measures, sucrose consumption and weight loss, to use in the pre-clinical study of dental pain. Further, it is clear that the

mechanisms for both sucrose consumption and weight loss are complex, but lend insight into the effects of pulpal injury on the quality of life of a mouse (Figure. 7). Surprisingly, unlike in the rat, measures of feeding behavior are unlikely to be useful in monitoring dental pain in mice.

Acknowledgements

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Figure 1.

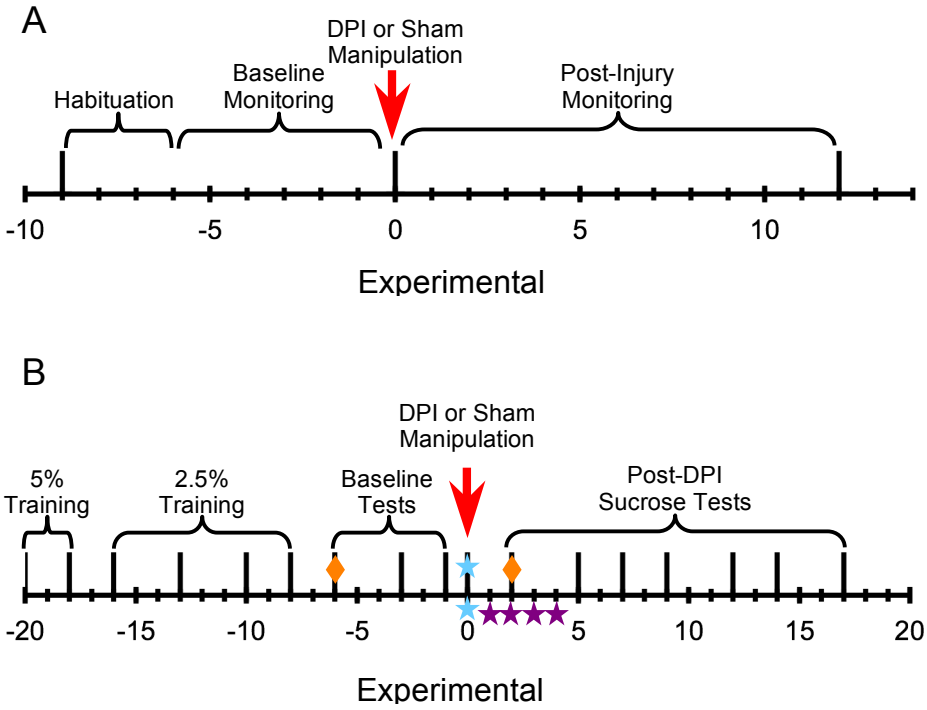


Figure 1. Timeline of experiments. (A) Home cage monitoring experiment proceeded with 3 days of habituation, 6 days of baseline monitoring, and 12 days of post-manipulation monitoring. (B) Timing of sucrose consumption test for room temperature tests (cold tests included an additional two training sessions before this timeline began.) Each black bar indicates a sucrose assay. The red arrow indicates the day of DPI/sham manipulation (always in the morning). The orange diamonds (♦) show the timing of naloxone injections and the stars show the timing of indomethacin injections, with blue stars (★) indicating injections of 3mg/kg and purple stars (★) indicating injections of 5mg/kg.

Figure 2

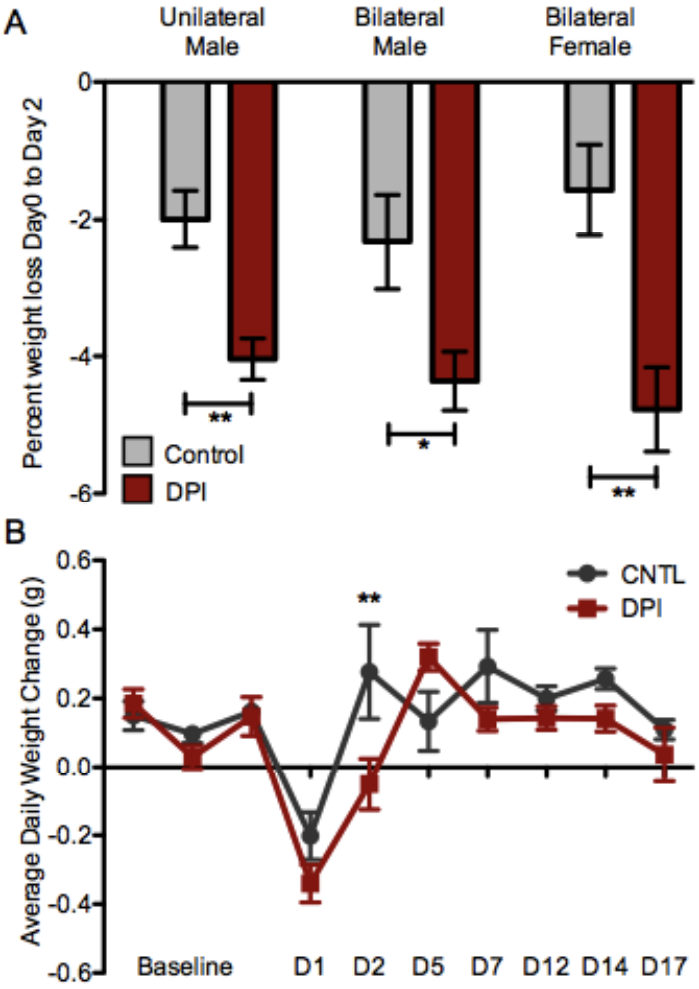


Figure 2. (A) Weight loss over the first two days after DPI, measured by percent change from original weight the morning of drilling in male mice with unilateral (n=7-8) and bilateral (n=30) DPI as well as females with bilateral DPI (n=8-10) (B) Average daily weight change on the three baseline days and each day after DPI (Two-way ANOVA, effect of treatment, p=0.001) CNTL: Control.

Figure 3.

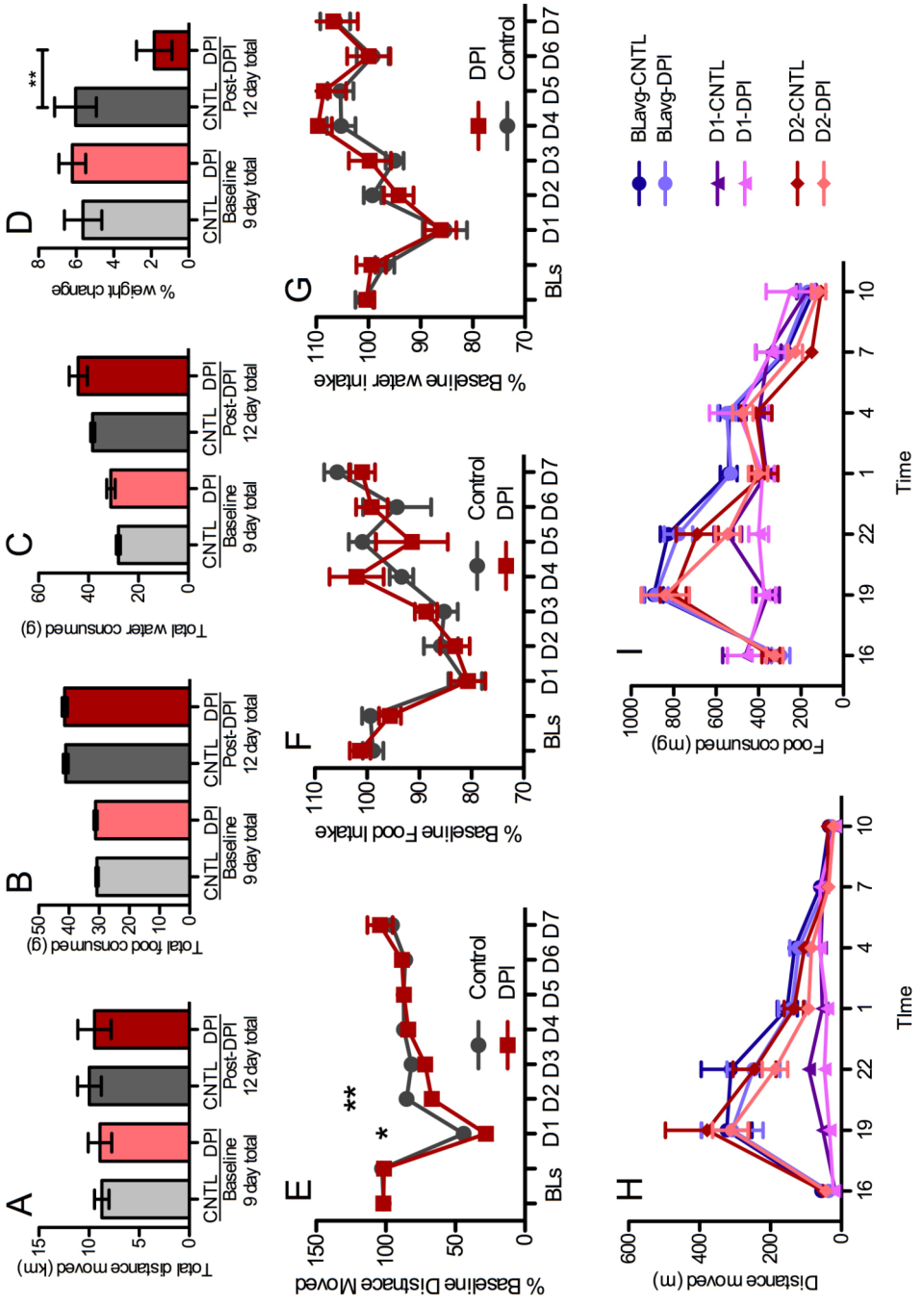

A
Total distance moved (km)
CNTL DPI
Baseline
9 day total
CNTL DPI
Post-DPI
12 day total
B
Total food consumed (g)
C
Total water consumed (g)
D
% weight change
**
E
% Baseline Distance Moved
Control
DPI
BLs D1 D2 D3 D4 D5 D6 D7
F
% Baseline Food Intake
G
% Baseline water intake
H
Distance moved (m)
Time
I
Food consumed (mg)
BLavg-CNTL
BLavg-DPI
D1-CNTL
D1-DPI
D2-CNTL
D2-DPI

Figure 3. The effect of DPI and sham manipulation on home cage behaviors. Total movement (A), food (B) and water (C) in the 9 days prior to manipulation (light grey and red bars) and 12 days after (dark grey and red bars) in animals that received sham (grey) or DPI (red). There were no significant differences between the two groups either before or after manipulation. (D) In the 12 days after DPI, mice gained less weight compared to controls and the 9 days prior to manipulation. (E-G) Average daily movement (E), food (F), and water (G) decreased as a result of anesthesia. There was an additional significant decrease in locomotion on days 1 and 2 after DPI. (H-I) Circadian pattern of locomotion (H) and feeding (I) was similar during baseline days (blues) and day 2 (reds) after DPI (dark lines) or control (lighter lines), but was altered on the first day (purples) after manipulation. CNTL: control

Figure 4.

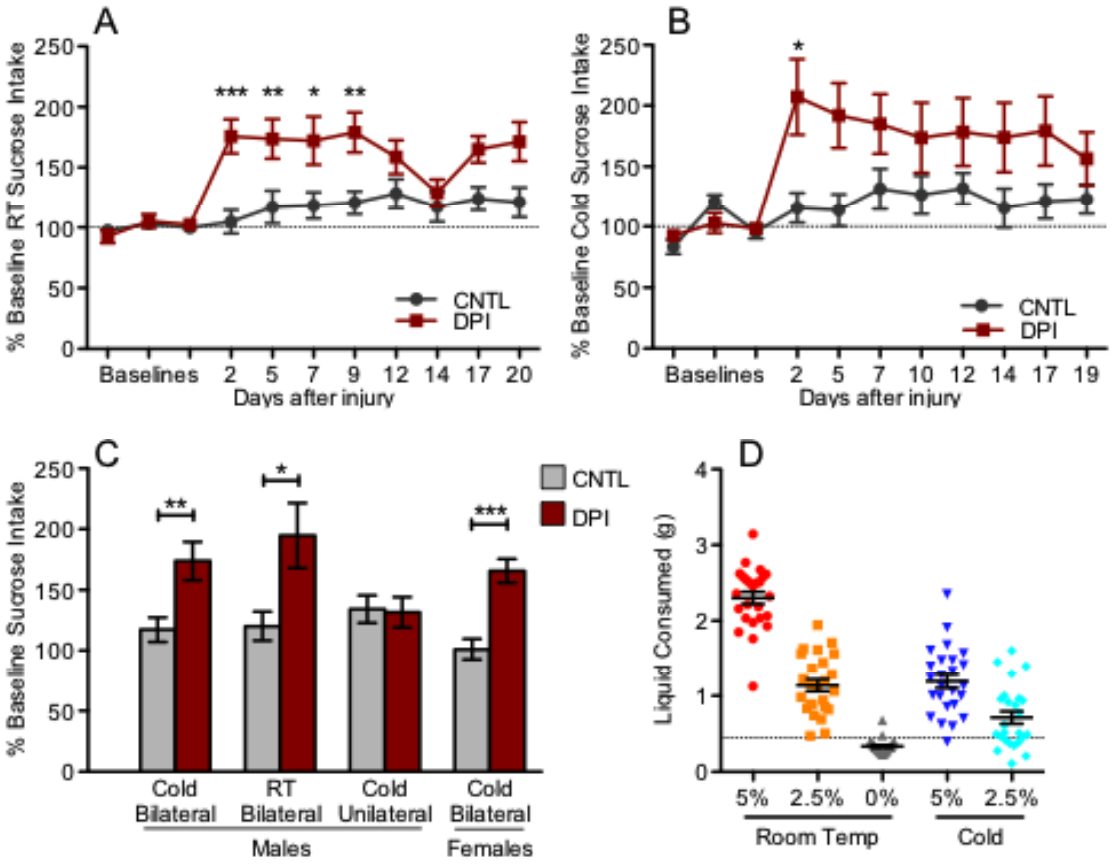

A
% Baseline RT Sucrose Intake
250
200
150
100
50
0
*** ** * **
CNTL
DPI
Baselines 2 5 7 9 12 14 17 20
Days after injury
B
% Baseline Cold Sucrose Intake
250
200
150
100
50
0
*
CNTL
DPI
Baselines 2 5 7 10 12 14 17 19
Days after injury
C
% Baseline Sucrose Intake
250
200
150
100
50
0
** * ***
CNTL
DPI
Cold Bilateral
RT Bilateral
Cold Unilateral
Cold Bilateral
Males
Females
D
Liquid Consumed (g)
4
3
2
1
0
5% 2.5% 0% 5% 2.5%
Room Temp
Cold

Figure 4. Sucrose consumption in mice with DPI. Mice with bilateral DPI (red lines) drank more sucrose after injury than control mice (grey lines) of either room temperature (A) or cold (B) sucrose. Data is normalized to the each mouse's baseline average and individual baseline days are also shown compared to the average of all baselines, to indicate the consistency of baseline consumption.. (C) The average sucrose consumption for the first week after injury (day 2-7) normalized to baseline consumption is increased in male and female mice with bilateral DPI in either cold or room temperature sucrose tests. Male mice with unilateral DPI did not increase intake compared to controls. (D) Sucrose consumption in naïve male mice indicates a concentration and temperature dependence on baseline motivation to drink during the two-hour test. Dotted line indicates the level below which animals are excluded from the DPI studies.

Figure 5.

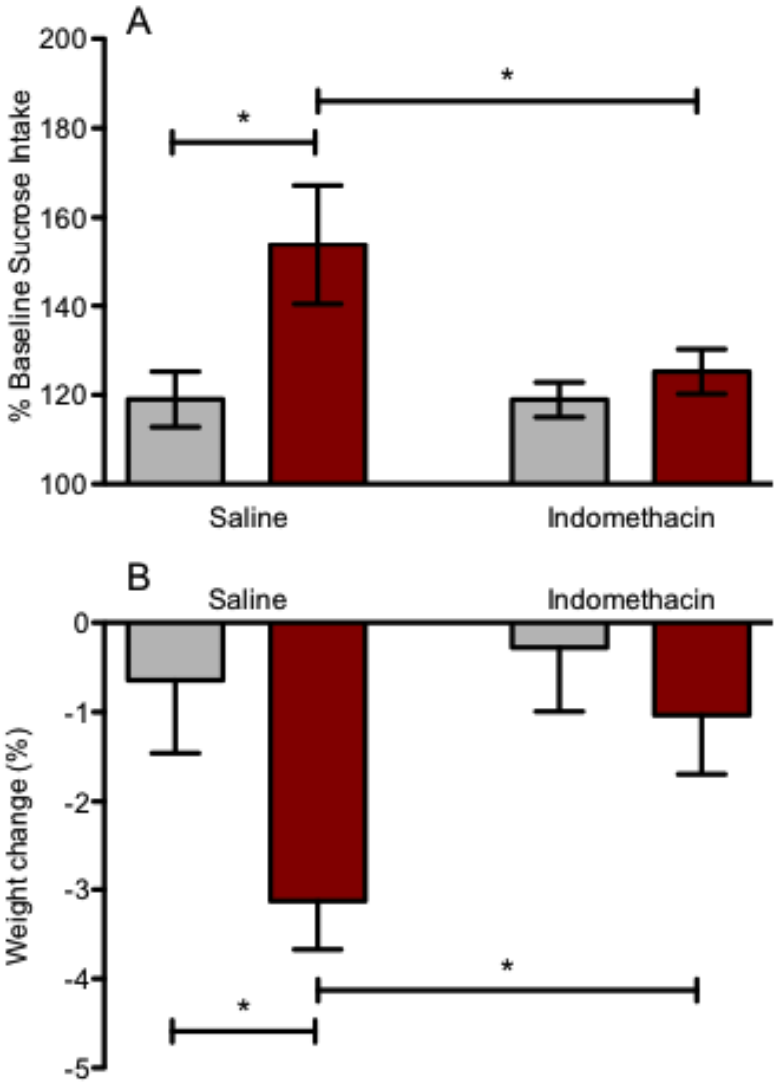


Figure 5. (A) Daily injections of indomethacin in the first 2 days after DPI prevent the injured animals from increasing sucrose intake as measured on day 2 after manipulation (one-way ANOVA: $F=4.3$, $p=0.01$, $n=11$-12). (B) Indomethacin also prevented DPI animals from losing more weight compared to controls (one-way ANOVA: $F=3.5$, $p=0.02$).

Figure 6.

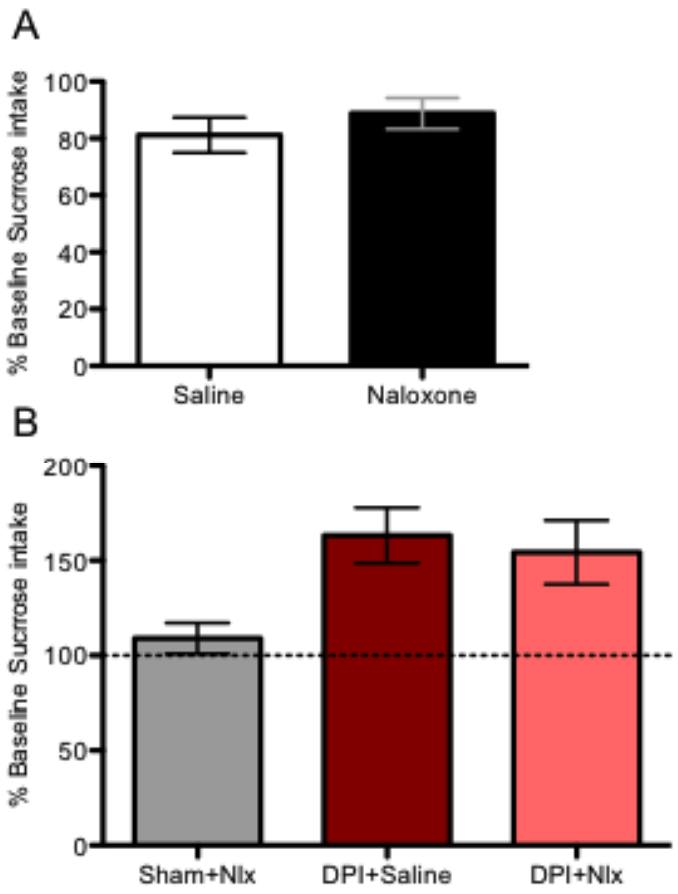


Figure 6. (A) Injections of naloxone (1mg/kg) 30 minutes prior to the sucrose consumption test in baseline animals did not significantly alter sucrose intake compared to saline injected animals. (p=0.39, saline: n=9, naloxone: n=16) (B) Naloxone given 30 minutes prior to testing on day 2 after manipulation did not prevent the DPI-induced increase in sucrose consumption. (one-way ANOVA, F=4.4, p=0.03, n=8-9).

Figure 7.

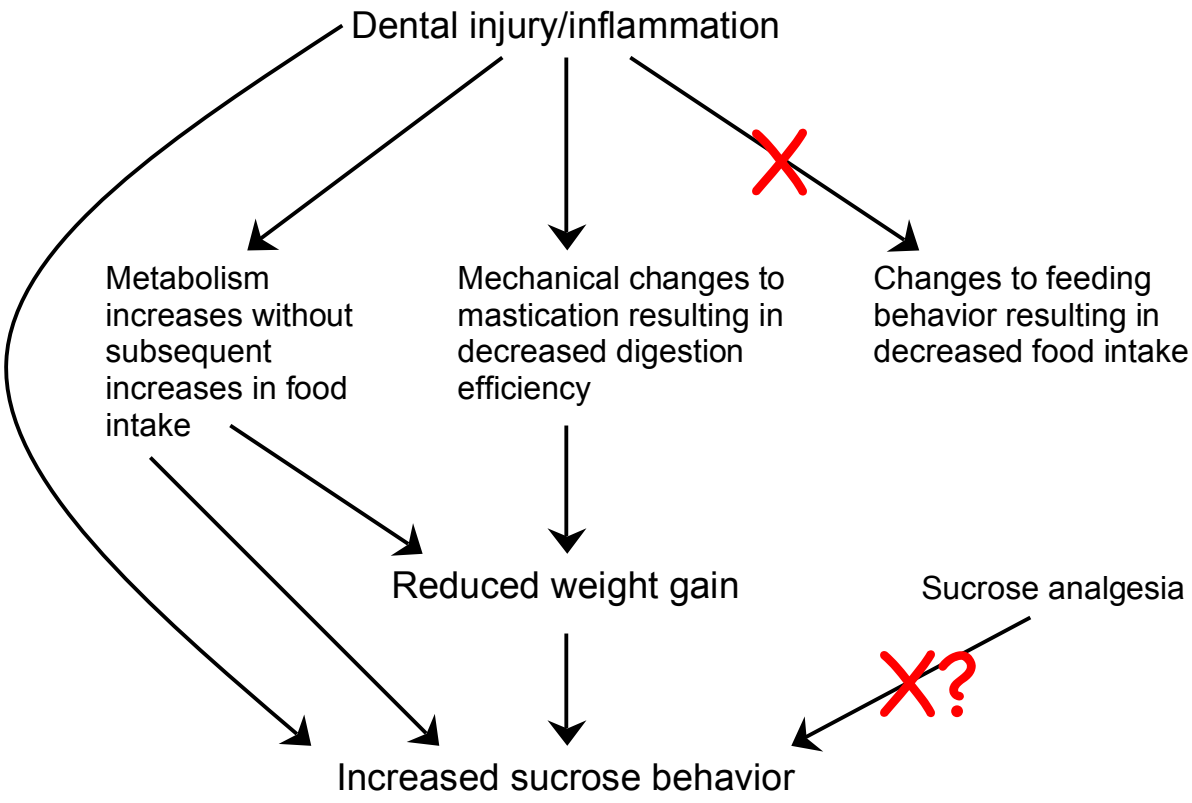


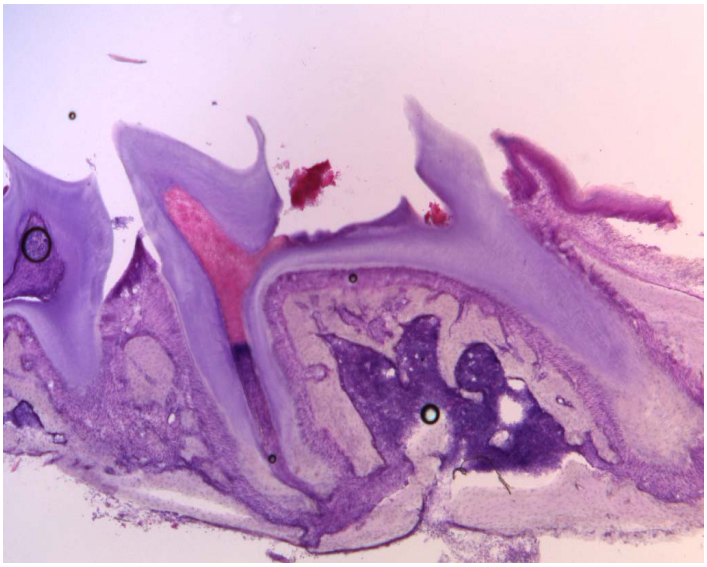
Figure 7. Model of relationship between weight loss, sucrose behavior and dental pulp injury. Red X's indicate relationships that have been excluded.

Table 1

		Control			DPI		
Feeding Bouts Averages		Baseline average	Day 1	Day 2	Baseline average	Day 1	Day 2
	Bout Number	90.8±9.2	60±8.3	74.5±7.7	106.2±28	54±10.9	95±21
	Bout Duration (s)	55±3.8	78.3±7.1	64.3±7.6	47.8±3	79.2±13.4	56.7±5.8
	Bout Size (mg)	39.3±4.4	50.3±5.3	38.8±4.4	40.3±7.9	62.7±8.1	35.5±5.5
	Intensity (mg/s)	0.71±0.06	0.65±0.05	0.61±0.04	0.84±0.14	0.81±0.05	0.61±0.04
Movement Bout Averages							
	Bout Number	4628±1071	1962±289	3848±667	4269±463	1385±281	2994±353
	Bout Duration (s)	1.3±0.1	1.47±0.12	1.34±0.11	1.48±0.1	1.53±0.06	1.48±0.07
	Bout Size (cm)	17±1.1	15.4±0.9	17.1±1.4	26.4±7.5	14±0.9	23.1±5.4
	Speed (cm/s)	13±1.3	10.5±0.4	12.5±0.9	13.1±1.4	8.9±0.4	12.5±1

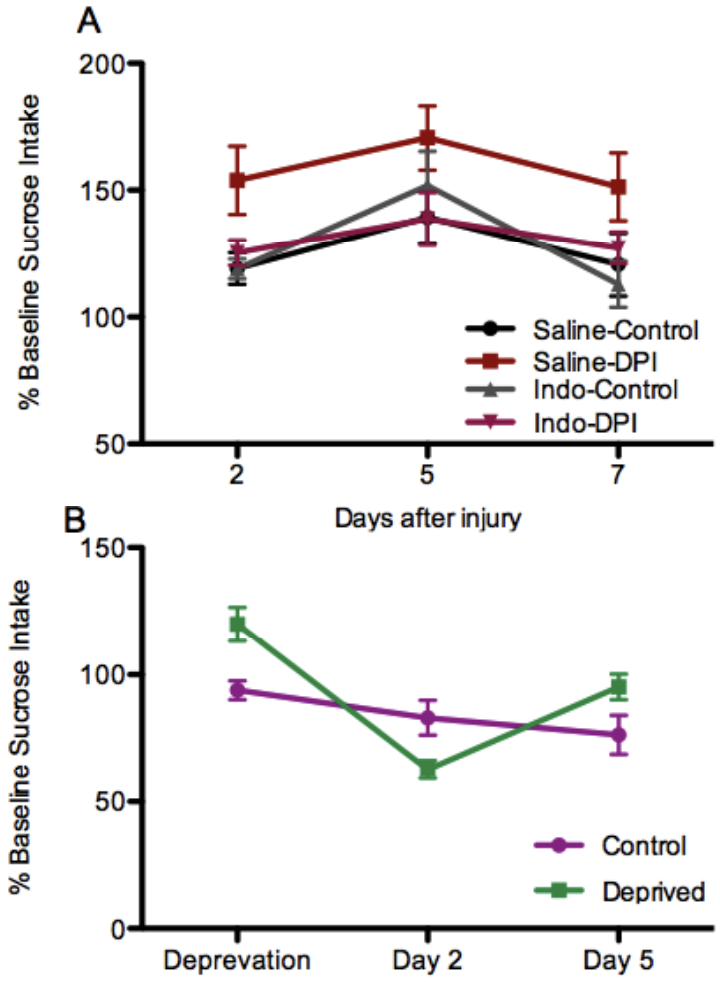
Bout averages in DPI and control animals in the first 48 hours after manipulation.

Supplemental Figure 1



Supplemental Figure 2. Hematoxylin and eosin stain of an injured tooth 17 days after initial pulp exposure. The extent of the initial drilled area can be seen and evidence of root necrosis is present (dark purple area).

Supplemental Figure 2



Supplemental Figure 2. (A) Daily indomethacin injections on days 0-4 affects DPI-related sucrose behavior on days 2-7. (Two-way ANOVA, effect of group $F=3.1$, $p=0.035$, $n=11$-12). Sucrose intake in the DPI-indomethacin group, did not subsequently increase intake after injections ended on day 4. (B) After a training period, a separate group of mice were food and water deprived for 12 hours immediately preceding a sucrose test using 2.5% room temperature sucrose. The deprivation resulted in increased sucrose intake, but two days later sucrose intake was significantly below controls. By day 5 after deprivation, intake had returned to baseline levels similar to control animals.

Chapter 5

Conclusions, Implications and Caveats

The use of mouse models in many fields of neuroscience, including pain, has risen sharply since the development and ease of genetic manipulation in the species. Inarguably, the mouse has been vital to many advances in mechanistic understanding of emotion, nociception and many other behavioral neuroscience fields. However, its use often raises as many problems as it solves (Kalueff et al., 2007; Mogil et al., 2010a; Ramos, 2008). One of the biggest issues of modeling human clinical experience in rodents is finding meaningful and appropriate dependent variables. The work presented here demonstrates in the mouse the use— or, rather, lack thereof — complex behavioral measures in the setting of injury.

In chapter 2, home cage behavior and performance in a battery of affective and locomotor tests was assessed in mice with models of neuropathic or inflammatory pain. Remarkably, though these standard models of chronic pain are associated with profound mechanical hypersensitivity, the animals did not display any other changes to quality of life surrogate measures of pain. These results are in stark contrast to both the human response to chronic pain as well as the behavior of rats with similar models of pain.

Additionally, we observed behaviors in three other models of persistent pain: osteoarthritis, disc-degeneration, and dental pulp exposure. Results from the pilot study of the former two models suggest that mice with joint degeneration (and, potentially, joint pain) have unaltered locomotor behavior in a series of disability tests. Finally, we measured behavior in the setting of dental pulp inflammation, for which there is no standard method of measuring pain levels. As with neuropathic and inflammatory models, pulpal injury also did not impact behavior in the home cage. Instead, we used an operant assay of sucrose consumption as a measure of dental pain/inflammation in mice. Data from this task suggest that pain can, in fact, influence some elements of complex behavior. However its utility as a measure of pain may be limited until the drivers of the weight loss and increased sucrose intake can be identified.

As discussed in the introduction and chapter 2, many of the behaviors addressed here, particularly in the affective dimension, are well studied in the rat. While there are a few mouse studies showing that disability and affective state can be used to assess models of chronic pain (Inglis et al., 2008; Suzuki et al., 2007), there are at least an equal number confirming our finding (Hasnie et al., 2007). Indeed, recent work from Jeffrey Mogil and colleagues has focused on similar problems of assessing ongoing, spontaneous pain in mice. Measuring disability and spontaneous behavior after SNI and CCI they found no behavioral changes, except for a gait preference for the contralateral paw in animals with SNI, but not CCI (Mogil et al., 2010b). Moreover, in mice with the same OA model used in this work, they demonstrated a

similar lack of changes in locomotion assays of disability (Malfait et al., 2010). Therefore, it may be of future interest to pursue the question of what might contribute to the differences in behavioral outcomes in the two related species.

We have used a wide array of different behavioral tests, however, we cannot rule out the possibility that there is, in fact, a behavioral measure or different method of testing similar behaviors, which may have yielded positive results. Even given the consistency in the lack of behavioral changes with these pain models over many paradigms, behavior can be sensitive to many methodological considerations (Chesler et al., 2002a, b; Rice et al., 2008; Schellinck et al., 2010). For example, of relevance here is the large effect on the behavior lighting can have in open field behavior, often causing paradoxical results at different levels of illumination (Strekalova et al., 2005). Even seemingly trivial changes, such as the type of bedding, can alter the behavioral outcome of pain at both the hypersensitivity and complex level (Robinson et al., 2004; Tokunaga et al., 2007). Therefore, we must acknowledge the possibility that these conclusions are only limited to the small parameters utilized here.

Further, a major caveat to this work, as well as of other mouse pain studies, is the length of time the animals experience "chronic pain." Typically in the clinic, a persistent pain lasting anywhere from 3 months to one year is considered chronic. Further, many chronic pain conditions last well over a year, with nearly 30% of pain respondents in one study having pain duration of over 15 years (Breivik et al.,

2006). But in the laboratory, waiting 6 to 12 months for the development of a chronic pain model in a mouse is impractical, not to mention unethical. Given the life expectancy of a mouse, which is on average 2-3 years in the lab, an injury that has an effect that lasts over a month may be the equivalent to several years in a human. Nonetheless, the shortened time period of "pain" may mean that the affective and quality of life changes that occur after years of suffering in humans simply do not have a chance to develop in the mouse models. Indeed, there is evidence that in rats some behavioral changes and concurrent prefrontal cortex activity changes do not occur until nearly 5 months after SNI (Seminowicz et al., 2009).

A second consideration in the use of animal models is that in doing so one is making a repeatable, predictable model of an inherently variable and subjective phenomenon. Nearly every animal with a nerve injury displays a subsequent mechanical hypersensitivity, while in humans, this is rarely the case. Pain in humans is far less predictable. For example, the correlation between radiographic evidence of osteoarthritis and the symptomatic disease is rather weak (Dieppe et al., 2005). Similarly, the incidence of chronic neuropathic pain after traumatic peripheral injury or diabetic polyneuropathy is 30-50% (Ciaramitaro et al., 2010; Van Acker et al., 2009; Werhagen et al., 2007). Compounded with this is the fact that the development of mechanical sensitivity only occurs in 56% of people with neuropathic pain (Maier et al., 2010). This discrepancy between the infrequency of pain after injury in the human compared to the reproducible hypersensitivity in

rodents — in addition to the lack of spontaneous measures of pain — suggests that key elements of the pain experience are lost in the translation to the simpler species.

Finally, the data presented here raises a number of key issues regarding the use of mouse models in the study of chronic pain. Most obvious is the implication that mice simply do not experience the persistent, ongoing pain to an extent that would affect quality of life measures. Alternately, given their status as small prey animals, they may be unable to display behavior suggestive of a chronic injury, despite experiencing an ongoing pain sensation. Though the lack of change in the open field test after the injection of LPS suggests the former, it is unclear which is actually the case. Regardless, the difficulty of finding behavioral measures of pain in mice is not surprising, in retrospect, given that gross observations of animals with these pain models rarely reveal noticeable differences. We are then left with the question of whether there is utility in continuing to model chronic pain in mice at all.

Though indisputably useful in the study of nociception and cellular mechanisms of nerve injury, the use of mice in the study of chronic pain maybe a difficult, if not impossible, endeavor. Indeed, there are those who have already suggested that the study of complex pain phenomenon may not be possible to study in rodents. In particular, Bud Craig is a proponent of the importance of the lamina I spinothalamocortical pathway to the dorsal posterior insula (dpIns) by way of the posterior part of the ventromedial nucleus (VMpo) in human pain (Craig, 2009). Craig argues that because a rodent does not have the needed forebrain structures

(that is, the VMpo and dpIns), it, therefore, lacks the necessary circuits to produce a human-like pain experience. While the existence of this pathway is subject to debate (Willis et al., 2002), our data appear to support this possibility.. Regardless of the exact mechanism at work, it may indeed be the case that mice simply lack ability to process pain in the same way as a human.

In general, the quest to perfect the use of animal models in pain and many other fields of behavioral neuroscience must walk a fine line between making the model relevant to the clinical condition and using ethologically relevant assays without anthropomorphizing the causes of an animals' behavior. But in the case of pain, by its very subjective nature, one must make assumptions about the "experience" of the animal. Here, we have used behavioral assessments of many of the same aspects of daily life that human clinical studies have used to assess pain (Chapter 1, Table 2), though in ethologically appropriate paradigms. The results here suggest that measures, which would reflect chronic pain, as opposed to simply a chronic change in nociceptive thresholds, do not change in models of neuropathy, inflammation, joint disease, and toothache. While this work stresses the need for careful consideration as to the limitations of mouse models in the study of chronic pain, it might also help to open new avenues of research if pain models can be found that alter the behaviors demonstrated here.

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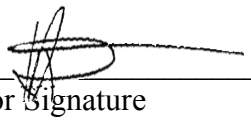

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