THE ROLE OF SLEEP AND CYTOKINES IN THE DEVELOPMENT OF MECHANICAL HYPERSENSITIVITY IN A MOUSE MODEL OF MUSCULOSKELETAL SENSITIZATION

by

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It is not the strongest of the species that survives,

nor the most intelligent that survives.

It is the one that is the most adaptable to change.

Charles Darwin

The best bridge between despair and hope is a good night's sleep.

E. Joseph Cossman

DEDICATION

This dissertation is dedicated to my mother,

Julie Sutton

Without her unwavering love and confidence

during the best of times and the darkest of days,

none of my accomplishments would be possible.

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CHAPTER I
INTRODUCTION

INTRODUCTION

The central nervous system (CNS) is essential for the survival of mammalian life. Two physiological functions of the CNS vital to that existence are sleep and pain sensation. Both encompass complex biological systems involving both the central and peripheral nervous systems to coordinate behavior with the external environment. Although the functions of sleep are not fully elucidated, sleep has a regulatory role in many homeostatic functions(1). Pain sensation is critical to protect organisms from external harm and ensure ongoing health. However, pain becomes pathological when acute pain sensation transitions to a chronic pain state. Characteristics of chronic pain include physical and/or emotional discomfort associated with tissue damage, and often persists after tissue heals(2). Sleep disorders in America impact an estimated 70 million patients and over 30% of the population reports insufficient sleep(3). Chronic pain also effects approximately 100 million Americans(4). The combined treatment costs of insufficient sleep and chronic pain are estimated in the trillions of dollars annually for the United States and represent substantial public health problems(3, 5). Sleep and pain have a bi-directional relationship; pain disrupts sleep quality and poor sleep enhances pain(6, 7). The immune system serves a regulatory role in physiological sleep and contributes to chronic pain, making it a

potential mechanism of interaction between sleep and pain(8, 9). The body of this dissertation investigates the relationship between sleep and chronic pain in a pre-clinical model of musculoskeletal pain with a focus on the role of cytokines.

SLEEP

Sleep, despite its ubiquitous nature, is one of the great unknowns in biology. Sleep is an essential function to maintain health, however 40% of the US population experiences at least one symptom of insomnia per year(10). Sleep is characterized as a behavioral state of decreased responsiveness that is rapidly reversible and quantified by the recording the electroencephalogram (EEG) and scoring for sleep state. Arousal states are broadly categorized into three stages; wakefulness, non-rapid-eye-movement (NREM) sleep, and rapid-eye-movement (REM) sleep. Sleep regulates homeostatic biological functions including pain and immune function. Conversely, challenges to the immune system and chronic pain impact the quality and quantity of sleep duration. For these reasons, the interrelationship among sleep, pain and the immune system are of interest.

The physiological mechanisms linking sleep and the immune system have been greatly elucidated during the last 40 years(9). Cytokines, small signaling molecules of the immune system, serve a functional role in sleep regulation under normal physiological and pathological conditions, including infection(11). Interleukin-1β (IL-1), IL-6, and tumor necrosis factor-α (TNF) are three cytokines that regulate the inflammatory response (discussed below). IL-1 and TNF are

sleep regulatory substances and IL-6 also modulates sleep(11, 12). Briefly, the roles of IL-1, IL-6 and TNF with respect to sleep are described.

IL-1 and TNF are cytokines that promote NREM sleep and induce symptoms associated with sleep loss(13). Administration of either IL-1 or TNF(14, 15), which mimics the normal accumulation of IL-1 and TNF across periods of wakefulness, induce cognitive impairment(16) and sleepiness(17); both symptoms of sleep loss. Sleep loss symptoms are diminished or blocked through the administration of cytokine inhibitors(18, 19). Protein and mRNA concentrations of IL-1 and TNF exhibit a diurnal variation in brain regions that correlates with sleep propensity(20, 21). After sleep deprivation, increases in NREM sleep, IL-1, and TNF expression occur in brain(22). Central injection of IL-1 or TNF, in addition to increasing the amount of time spent in NREM sleep, enhances the EEG delta (0.5 – 4 Hz) power (a measure of sleep intensity(23, 24)) during NREM sleep but not during wakefulness or REM sleep(15). Enhancement of delta power during NREM sleep is a characteristic of recovery sleep after prolonged wakefulness(25, 26). Collectively, these data support that IL-1 and TNF regulate sleep-wake behavior, especially NREM sleep.

IL-6 exerts effects on sleep. In humans IL-6 secretion is under circadian control, with serum concentrations being lower during the daytime and higher at night(27), and delaying sleep onset postpones IL-6 secretion(28). Administration of exogenous IL-6 increases NREM sleep in rats(29). Genetic loss of IL-6 using a ligand knockout mouse does not change baseline sleep behavior, but does alter the responses of mice to sleep deprivation(30). In healthy human subjects,

IL-6 administration increases NREM sleep and fatigue(31). Inhibition of IL-6 is a common treatment for rheumatoid arthritis and significantly reduces patient reported fatigue and improves subjective sleep quality(32, 33). These data support the modulatory role of IL-6 in sleep-wake behavior.

Disordered sleep is an escalating problem as sleep duration and quality decline(3). Causes of disordered sleep include social distractions (broadly termed as 'social jetlag'(34)) and increases in light availability(35). Experimental sleep restriction and deprivation in humans cause insulin resistance and increase caloric consumption, diminish immune response, impair decision-making, reduce attention span, reduce cognitive speed, and decrease tolerance to painful stimuli(36-45). Epidemiological evidence demonstrates that in humans habitually short sleep duration increase the incidence rates of obesity, diabetes, glucose intolerance, cardiovascular disease, susceptibility to infection, and chronic pain(46-55). Short sleep duration is also linked to an increase in risk of all-cause mortality(56). Although the biological mechanisms underlying increases in disease risk are the subject of ongoing research, short sleep duration is a contributing factor to disease states including chronic pain and immune function.

The functions of sleep are studied through the experimental deprivation of sleep in humans and rodents. Methods of total sleep deprivation eliminate both NREM and REM sleep(57). REM sleep deprivation selectively targets REM sleep for elimination while leaving NREM sleep intact(58). These techniques yield data from humans and rodents, however sleep deprivation does not model the human condition of disrupted sleep. Sleep disruption has many causes

including sleep disorders, chronic medical conditions, shift work, and pain(7, 59, 60). Characteristics of sleep disruption include brief arousals during sleep that do not reduce total sleep time(61), but increase daytime sleepiness and impair cognitive function(62). Sleep disruption of humans and rodents increases concentrations of circulating cytokines including IL-1, IL-6, and TNF(63, 64). Interest in modeling the human condition of disrupted sleep led to the development of sleep fragmentation (SF) techniques(65, 66) that fragment sleep without reducing overall sleep time. One method validated by our laboratory fragments the sleep of mice for prolonged periods of time using a rotating disc(65). The use of this device during the light period to disrupt sleep is thought to more closely model the human condition of sleep disruption(65). For these reasons this dissertation will utilize SF to investigate the impact of sleep disruption on subsequent pain and sleep behavior.

CYTOKINES

The immune system is broadly divided into the innate immune system, which encompasses numerous antimicrobial mechanisms(67), and the acquired immune system that uses antibodies and cytotoxic cellular mechanisms(68). One of the primary functions of the innate immune system is to recruit immune cells to the site of the infection to segregate biological threats for removal(68). The innate immune system isolates and destroys invading pathogens through antigen recognition and inflammatory processes(67). The innate immune system response activates major effector cells, which include neutrophils, monocytes, natural killer cells, natural killer T lymphocytes, and γδ T cells, all of which

release cytokines(69). Cytokines are specialized mediators of the immune system that signal during normal physiological and pathological conditions.

Cytokines are small (8-30 kilodaltons) molecules used by the immune system for endocrine and paracrine signaling (70). The term cytokine applies to over 100 proteins, peptides, and glycoproteins that signal and recruit immune cells to the site of an infection. Cytokines are broadly divided into two major categories: pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines include IL-1, IL-6, and TNF; three of the most extensively studied cytokines. IL-1, IL-6, and TNF are produced from virtually all nucleated cells, especially resident macrophages, endothelia, and epithelial cells. IL-1, IL-6, and TNF trigger inflammatory cascades that contribute to a number of physiological and pathological processes, including sleep and pain(13, 71). The function of each of these cytokines in the immune system is briefly discussed below.

IL-1 is an inflammatory cytokine with a size of ~ 36 kDa that is created when pro-IL-1 is cleaved into active form by caspase-1(72, 73). IL-1 is produced in response to an injury, infection or immune challenge(74). IL-1 causes fever and contributes to the production of other proinflammatory cytokines(74, 75). Two receptor subtypes bind IL-1; IL-1 receptor 1 (IL1-R1) and IL-1 receptor 2 (IL1-R2). IL1-R1 interacts with a cytoplasmic accessory protein to form a complex that recruits adaptor molecules(76). This signaling activates the transcription factor NF-κB that in turn triggers complex intracellular cascades(69). IL1-R2 lacks an intracellular domain and as such acts as a decoy receptor(77,

78). Foreign organisms and their byproducts stimulate IL-1 production(79), as well as cytokines including TNF(80), and IL-1 itself(80, 81).

TNF activates inflammatory response cascades and was first identified as a product of macrophages and lymphocytes that lyses cells, especially tumor cells, conferring its name(82). TNF is synthesized as a membrane-bound homotrimer, pro-TNF, that is cleaved by the TNF converting enzyme and creates the soluble cytokine that interacts with cell-surface receptors(83, 84). TNF, like IL-1, has two receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2), which are structurally related but differ with respect to their affinity for ligands and cellular expression(84). Both of the TNF receptors signal through transcription factors, including NF-κB(85). The activation of these transcription pathways is critical in the immune response, release of cytokines, and induction of cell apoptosis and necrosis(86).

Interleukin-6 (IL-6) is a protein critical in the maturation of B cells into antibody-producing cells(87). IL-6, in addition to differentiating B-cells, contributes to the regulation of other immune cells, hepatocytes, and the skeletal, cardiovascular, endocrine, and nervous systems(88). Neurons, astrocytes, microglia, and endothelial cells produce IL-6, especially after injury(89). IL-6 binds to class I cytokine receptors that include gp130 which is ubiquitously expressed(88). The specific receptors for IL-6 comprise a soluble (sIL6R) and membrane bound (mIL6R) receptor whose expression are restricted to specific tissues(90). Upon recruitment of gp130 after binding of IL-6, intracellular signaling is activated(91). Changes in the gene transcription of NF-κB regulate

IL-6 secretion, although post-transcriptional mechanisms also regulate IL-6 secretion(92, 93). IL-6 is an inflammatory cytokine although it also exhibits indirect anti-inflammatory effects by inhibiting TNF and IL-1 via release of IL-1 receptor antagonist and IL-10, an anti-inflammatory cytokine(94, 95). IL-6 is also considered to be a myokine, or a cytokine directly released from muscle tissue, especially after muscle contraction(96, 97).

Cytokines serve a functional role not only in immune responses, but also sleep and pain. The role of cytokines in the regulation of sleep, especially NREM sleep, is already touched upon. Briefly, the roles of cytokines in acute and chronic pain are discussed below.

PAIN

Chronic pain is the most prevalent disease in the United States with 100 million American's reporting chronic pain during their lifetime(4). Unfortunately, over 75% of persons with chronic pain will have insufficient control over their pain(4). These numbers contribute to the $675 billion in annual costs associated with medical treatment and lost work productivity related to chronic pain(5). Three of the most prevalent types of chronic pain in the United States are musculoskeletal in nature and include low back pain, neck pain, and facial pain(4). The pervasiveness of chronic musculoskeletal pain supports the need for research, especially pre-clinical research, to elucidate the biological mechanisms underlying muscle pain and develop effective treatments(4).

There are many causes of chronic pain. A direct injury or insult to the central nervous system causes neuropathic pain and often results from surgery

or traumatic injury that can crush, tear, or sever the nervous system. Tissue inflammation activates the immune system and causes inflammatory pain. Damage to the joints and connective tissues causes rheumatic pain and is often progressive in nature, such as during osteoarthritis. Musculoskeletal pain is associated with conditions including fibromyalgia and a number of painful myopathies. Musculoskeletal pain is important not only because of its frequency, but also the escalating costs of treatment(98, 99). The most prevalent workplace injuries involve musculoskeletal pain, and pain control is the biggest barrier in returning to work(100). Furthermore, control of musculoskeletal pain is often poor and may require the ongoing use of opioids that poses risks of dose escalation to achieve pain control and drug dependence(101). Patients with musculoskeletal pain also have a poorer quality of life compared to patients with chronic gastrointestinal conditions, renal disease, and cardiovascular conditions(102). The study of musculoskeletal pain is necessary to reduce economic costs, identify treatments, and improve patient quality of life.

Nociception is the process of detecting a physical threat in the form of mechanical, chemical, or thermal stimuli that are of a sufficient intensity to produce tissue damage(103, 104). Sensing noxious stimuli is different from non-noxious physical sensation of the external environment and uses unmyelinated c fibers and thinly myelinated Aδ fibers, generally termed nociceptors(2). Nociceptors detect noxious sensations including harmful cold, heat, pressure, chemicals, and hydrogen ions (H^+) and synapse from the periphery into the dorsal horn of the spinal cord(2). The dorsal horn integrates, processes, and

outputs sensory information from the spinal networks that project to the brain in several discrete pathways that detect the discriminative and emotional aspects of pain(105). These ascending pathways further relay information on to the cortex. The ascending pain pathways, from the periphery to CNS, mediate the basic sensation of acute noxious stimuli.

Descending pathways from the brain to the spinal cord facilitate and inhibit pain sensation(106). Inhibitory mechanisms have evolutionary importance because they enable an organism to suppress pain in situations that may require 'fight or flight' to survive(107). Descending pain pathways include the hypothalamus, amygdala, and cortex that send projections to the midbrain periaqueductal gray (PAG) region. The PAG outputs synapse in the medulla within the rostral ventromedial medulla (RVM) and project to the dorsal horns of the spinal cord(108). A large body of research from pharmacological, electrophysiological and anatomical studies identifies the periadueductal gray (PAG) and rostroventral medulla (RVM) as being critical nuclei in regulating sensory processing in the dorsal horn(108-110). Chronic pain can impair descending inhibition of pain.

Central sensitization is a term that broadly describes the augmentation in nociceptive pathway functions during chronic pain(111). The neural plasticity associated with central sensitization occur at diverse anatomical (molecular, cellular, synaptic, and network) and temporal (acute to chronic) scales(112). Central sensitization leads to pre- and post-synaptic potentiation through regulation of neurotransmitter release and receptor expression(111).

Characteristics of central sensitization include increases in neuronal excitability, both spontaneous and evoked, the expansion of nociceptor receptive fields, and the reduction of threshold for nociceptor action potentials(113). Structurally, synaptic spine density can increase or decrease during chronic pain. Denervation and hypertrophy can enhance pain, despite having converse effects on cellular connections(111). Modulation of cell numbers in brain gray matter, cell death and cell proliferation in the spinal cord all are associated with chronic pain states(113). Trends in regulation of structures involving nociceptive transmission are emerging, although the specific changes during chronic pain differ across research models.

Musculoskeletal sensitization models muscle pain in pre-clinical rodent models. Musculoskeletal sensitization uses two injections of acidified saline (pH 4.0) unilaterally into the gastrocnemius muscle spaced 5 days apart to produce long lasting bilateral secondary mechanical hypersensitivity at the hindpaws(114-116). Mechanical hypersensitivity is measured by quantifying hindpaw withdrawals to calibrated pressure von Frey monofilaments(117). The musculoskeletal sensitization model does not cause classical signs of pain including weight bearing preference, impaired locomotor function, guarding of the limb, or limping(114). The bilateral mechanical hypersensitivity at the hindpaws during musculoskeletal sensitization is the result of changes in the CNS, not the periphery(118, 119). This makes musculoskeletal sensitization an ideal model to study interactions with sleep, a process mediated entirely by the CNS. Peripheral tissue damage or impairment of locomotor function, as seen during

pre-clinical models of osteoarthritis and neuropathic pain, make interpreting the relationship between sleep and pain challenging as changes in sleep could result from limited mobility. The lack of peripheral tissue damage allows for a targeted interaction of sleep with musculoskeletal sensitization. Musculoskeletal sensitization in mice is utilized in this dissertation to investigate mechanistic interactions between musculoskeletal pain and sleep.

PAIN AND CYTOKINES

The activation of the inflammatory process is classically associated with pain (dolor), redness (rubor), swelling (tumour), heat (calor), and loss of function (function laesa). Cytokines are part of the inflammatory immune response and contribute to proper wound healing at the site of injury and infection(120). However, excessive or prolonged inflammation can interfere with normal homeostatic function and contribute to pathological pain states(8). IL-1, IL-6, and TNF all serve functional roles in the inflammatory response following injury, infection, and modulate pain(121). Briefly, the role of each individual cytokine with respect to pain is reviewed.

IL-1 is primarily produced by macrophages, monocytes, and non-immune cells including activated fibroblasts during cellular damage, inflammation and infection(75). IL-1 produces systemic inflammation through activation of the cyclooxygenase-2 pathway that is commonly targeted for pain reduction through the use of non-steroidal anti-inflammatory (NSAID) drugs(71). IL-1 also activates the production of substance P (SP), nitric oxide, and endothelial adhesion molecules, all of which contribute to the development and maintenance of

pain(71, 122). Dorsal root ganglion cells of the spinal cord express IL-1 receptors in nociceptive neurons(71, 123), providing a direct mechanism for pain enhancement(124). Central or peripheral injection of IL-1 produces lasting pain in rodents(125). Furthermore, antagonism of IL-1 prevents the development of pain in an experimental model of neuropathic pain(126, 127). Collectively, these data support the role of IL-1 in pain.

TNF is one of the first cytokines identified, along with IL-1, to cause pain(128). In a variety of clinical conditions, including fibromyalgia and rheumatoid arthritis, TNF is elevated in patients(129). Increases in cytokine concentrations correlate with augmented pain for patients with chronic pain conditions(130). Administration of TNF using injection or topical application produces pain in pre-clinical models(131-135). The painful effects of TNF are reversible through the co-administration or pre-treatment with TNF receptor antagonists in models of neuropathic pain and diabetic pain(136-138). Clinically, these findings are reproduced using pharmaceutical TNF receptor antagonists and inhibitors for the treatment of pain caused by rheumatoid arthritis(139, 140). Collectively, these data support a role of TNF in the regulation of pain.

IL-6 is a cytokine critical to the acute phase response of the innate immune system(89, 94). IL-6 is released during trauma, infection, injury, surgery, burns, and strenuous exercise(89). IL-6 is considered to be one of the most relevant markers of tissue damage(141), however it’s role in pain is not as well elucidated as IL-1 and TNF. IL-6 is an excellent candidate molecule for relaying information between the periphery and central nervous system(142). Gp130, a

critical component of IL-6 signaling, is implicated in numerous disease states(88). Gp130 amplifies pain through the induction of cytokine release, and genetic knockout of gp130 in mice significantly reduces pain to noxious stimuli(143). Importantly, IL-6 is an activator of gp130 and downstream signaling cascades contributing to the development of pathological pain(143). Blockade of the IL-6/IL-6R/gp130 formation is a therapeutic treatment for patients with rheumatoid arthritis, multiple myeloma, and Castleman's disease(144, 145). Antagonists for IL-6/gp130 are approved by the FDA for treatment of patients not helped through anti-TNF therapy(146-148). These data demonstrate the importance of IL-6 as a pathway for the modulation of chronic pain.

Cytokines are critical in the acute and chronic pain response. Although the role of cytokines under specific chronic pain states is still being elucidated, cytokines contribute to pain processing. The administration of cytokines to produce pain, the blockade of cytokines to relieve pain, and the quantification of cytokine protein and gene expression increases following trauma, inflammation, and injury demonstrate the role of cytokines during pain.

SLEEP AND PAIN

Sleep and pain have bi-directional interactions, with pain disrupting sleep and poor sleep enhancing pain(6, 149). Experimental and observational work in clinical and pre-clinical models supports this relationship. Despite this body of work, pre-clinical models have not investigated interactions between sleep and musculoskeletal pain. The need for research between sleep and musculoskeletal pain is underscored by the epidemiological data demonstrating

large numbers of persons suffering from musculoskeletal pain, insufficient sleep, or a combination of these chronic conditions(3, 4, 7).

Clinically acute and chronic pain interfere with sleep(7). Early studies systematically investigating the role of pain on sleep quantified the sleep of patients with chronic musculoskeletal, rheumatic, and non-specific pain(150-152). All of these patients had diminished sleep quality characterized by sleep fragmentation, reduced NREM sleep, reduced REM sleep, and reduced overall sleep time(150-152). Epidemiological data reveal that sleep disturbances, especially insomnia, are the highest associated comorbidity with chronic pain, with rates ranging between 50 and 98%(60, 153, 154). Sadly, the negative influence of chronic pain on sleep quality holds true for children and adolescents with chronic pain(155, 156). Although this research is only briefly touched upon, there is a substantial body of literature demonstrating the negative role of chronic pain on sleep quality consistent with data presented(157).

The influence of pain on sleep is pre-clinically studied through the use of animal models of pain(158, 159). Pre-clinical models investigate osteoarthritis, nerve injury, inflammatory pain, and immune related pain(158). A number of pre-clinical chronic pain models are associated with decrements in sleep quality(160-167). Changes in sleep are characterized by a reduction in total sleep time, NREM and REM sleep time individually, fragmentation of the sleep period, increases in latency to sleep, and changes in EEG characteristics(160-167). Pre-clinically, rodent models of chronic pain and sleep support the clinical evidence that pain disrupts sleep. One gap in the current literature is the influence of

musculoskeletal pain on sleep. Although pre-clinical models of musculoskeletal pain are available, none have been investigated with respect to sleep(114, 134). This presents an opportunity for pre-clinical research into the influence of musculoskeletal pain on sleep.

Sleep and pain research also investigates the role of sleep in modulating pain sensation(168). Acute and chronic pain is the highest associated co-morbidity with primary insomnia(169, 170). Numerous clinical studies demonstrate that experimental sleep deprivation, restriction, or disruption of human subjects enhances pain across sensory modalities(45, 171-173). In the general population, persons with a sleep durations outside the 6-9 hour range per night have increases in subjective pain(174). Restorative sleep is a predicting factor in the resolution of pain in patients with chronic widespread pain(175). Epidemiological data identifies poor subjective sleep quality as an independent risk factor in the development of fibromyalgia(55). This body of literature provides evidence that sleep quality and duration have a modulatory effect on pain perception.

Pre-clinical sleep disruption, restriction and deprivation of animals enhances pain independently, or in conjunction with chronic pain(176, 177). Sleep deprivation is performed through a number of methods either selectively targeting REM sleep or total sleep deprivation(66, 178, 179). REM sleep deprivation sensitizes rats to thermal stimuli and recovery sleep improves sensitivity(180, 181). Numerous studies demonstrate that REM sleep deprivation enhances pain across a range of modalities(182-185). Interest in modeling the human condition

has led to the development of animal models of sleep fragmentation(65, 66). These models are not as extensively studied for their role in pain as REM sleep deprivation because of their recent development. However, given the body of clinical literature linking poor sleep quality with enhanced pain in humans, pre-clinical studies should yield congruent results.

Sleep and pain have a bi-directional relationship. Acute and chronic pain disrupt and diminish the quality of sleep in humans and pre-clinical research models. The study of clinical patients with chronic pain or the pre-clinical induction of chronic pain using rodent models negatively impacts sleep. Conversely, poor sleep quality contributes to the enhancement and exacerbation of pain. Deprivation and disruption of the sleep of human subjects or rodents enhances pain in healthy subjects and those with pain. Furthermore, population based studies reveal that poor sleep quality is a risk factor for the development of chronic pain. Collectively these data support the influence of sleep and pain on each other and identify pre-clinical research of musculoskeletal pain as an area of research.

SPECIFIC AIMS

The central hypotheses of this dissertation are that 1) musculoskeletal sensitization will disrupt sleep, 2) cytokines will play a role in mechanical hypersensitivity following musculoskeletal sensitization, and 3) sleep fragmentation combined with musculoskeletal sensitization will alter the behavioral outcomes of musculoskeletal sensitization. Chapter II quantifies sleep/wake behavior of mice after musculoskeletal sensitization using EEG

instrumentation for determination of sleep physiology. Chapter III investigates the role of intramuscular cytokines in musculoskeletal sensitization. These experiments quantify inflammatory cytokines in muscle tissue after musculoskeletal sensitization, and use pharmacological and genetic tools to determine the roles of NF-κB, IL-1, IL-6, and TNF in mechanical hypersensitivity. Chapter IV investigates the role sleep fragmentation combined with musculoskeletal sensation has in altering behavioral outcomes including mechanical hypersensitivity and subsequent sleep. These hypotheses are derived from the literature that pain disrupts sleep, poor sleep enhances pain, and cytokines modulate pain. Elucidating these mechanisms will contribute to the basic biological understanding of mechanisms relating sleep and musculoskeletal pain.

CHAPTER II
MUSCULOSKELETAL SENSITZATION AND SLEEP: CHRONIC MUSCLE PAIN FRAGMENTS SLEEP OF MICE WITHOUT ALTERING ITS DURATION[1]

ABSTRACT

Study Objectives: Musculoskeletal pain in humans is often associated with poor sleep quality. We used a model in which mechanical hypersensitivity was induced by injection of acidified saline into muscle to study the impact of musculoskeletal sensitization on sleep of mice.

Design: A one month pre-clinical study was designed to determine the impact of musculoskeletal sensitization on sleep of C57BL/6J mice.

Methods: We instrumented mice with telemeters to record the electroencephalogram (EEG) and body temperature. We used an established model of musculoskeletal sensitization in which mechanical hypersensitivity was induced using two unilateral injections of acidified saline (pH 4.0). The injections were given into the gastrocnemius muscle and spaced five days apart. EEG and body temperature recordings started prior to injections (baseline) and continued for three weeks after musculoskeletal sensitization was induced by the second injection. Mechanical hypersensitivity was assessed using von Frey filaments at

[1] The work presented in this chapter are accepted for publication: Sutton, B.C. & Opp, M.R. (2013) Musculoskeletal sensitization and sleep: Chronic muscle pain fragments sleep of mice without altering its duration. SLEEP, in press.

baseline (before any injections) and on days 1, 3, 7, 14, and 21 after the second injection.

Results: Mice injected with acidified saline developed bilateral mechanical hypersensitivity at the hind paws as measured by von Frey testing and as compared to control mice and baseline data. Sleep during the light period was fragmented in experimental mice injected with acidified saline, and EEG spectra altered. Musculoskeletal sensitization did not alter the duration of time spent in wakefulness, non-rapid eye movement sleep, or rapid eye movement sleep.

Conclusions: Musculoskeletal sensitization in this model results in a distinct sleep phenotype in which sleep is fragmented during the light period, but the overall duration of sleep is not changed. This study suggests the consequences of musculoskeletal pain include sleep disruption, an observation that has been made in the clinical literature but has yet to be studied using preclinical models.

INTRODUCTION

It is estimated that 1.5 billion people worldwide suffer from moderate to severe chronic pain(186). Individuals suffering chronic pain comprise one of the costliest patient populations, especially in terms of lost work and reduced productivity(187, 188). Persons with chronic pain also report some of the lowest quality of life among patients suffering from chronic diseases(102). Poor sleep is another major public health issue, with almost 40% of the US population reporting chronic insufficient sleep, and 50-70 million Americans diagnosed with sleep disorders(3). Sleep disorders and chronic pain are often comorbid

conditions, and the overall prevalence and economic burden of chronic pain and insufficient sleep make these diseases an important topic of public health research.

Data derived from clinical research supports a bidirectional relationship between sleep and chronic pain(149, 176, 189). A variety of chronic pain conditions have comorbid sleep disturbances(60, 153, 169). Sleep of patients with chronic pain is characterized by difficulty initiating sleep, maintaining sleep, excessive nighttime awakenings, and feeling unrefreshed after sleeping(7, 60, 149, 176). For example, individuals suffering with chronic low back pain have insomnia rates over 50%, and subjective pain correlates with severity of insomnia(190). Persons with primary insomnia also report chronic pain at rates over 50%, the highest associated comorbidity for insomnia(191). Recent epidemiological research identifies a history of poor sleep quality as a significant risk factor in the development of fibromyalgia(55). Furthermore, experimental disruption or deprivation of sleep reduces pain thresholds(172, 176, 192). Conversely, extension of the sleep period is sufficient to reduce pain sensitivity, suggesting that sufficient sleep may reduce pain(193). Collectively, these and other data contribute to our understanding of the relationship between poor sleep quality and chronic pain.

Three of the most prevalent types of chronic pain in our society are low back pain, neck pain, and facial pain(4), all of which are musculoskeletal. The most prevalent chronic pain conditions associated with insomnia are arthritis (primarily rheumatoid), spinal pain (including low back pain), and

fibromyalgia(194-196). Although preclinical models of osteoarthritis(165, 197-199), sciatic nerve injury(161, 200-202), and inflammatory pain(203) have been used to determine the impact of chronic pain on sleep, none of these conditions constitute musculoskeletal pain. To the best of our knowledge, no preclinical models have been used to investigate the effect of chronic musculoskeletal pain on sleep. Changes in rodent sleep in models of osteoarthritis, sciatic nerve injury, and inflammatory pain include increased wakefulness, decreased rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep, and an increased latency to sleep onset(160, 161, 197, 199, 203). Given the clinical correlations between some musculoskeletal pain conditions and altered sleep, we hypothesized that musculoskeletal sensitization would disrupt sleep of rodents. To test this hypothesis, we quantified sleep of mice before and after musculoskeletal sensitization. We now report that musculoskeletal sensitization fragments the sleep of mice and alters some facets of the sleep EEG.

METHODS

Animals

Adult male C57BL/6J mice (22–25 g) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12:12 h light:dark cycle at 27°C with *ad libitum* access to food and water. All procedures using mice in these studies were approved in advance by the University of Washington Institutional Animal Care and Use Committee (IACUC), in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals.

Musculoskeletal Sensitization

Musculoskeletal sensitization using acidified saline injections has previously been used to study aspects of the muscle pain associated with chronic pain conditions, including chronic widespread pain and fibromyalgia, in rodents(114, 115). Briefly, this protocol involves 2 unilateral injections into the gastrocnemius muscle spaced 5 days apart of either normal (pH 7.2, control) or acidified (pH 4.0) saline. When acidified saline is injected using this protocol, a robust bilateral secondary mechanical hypersensitivity at the hindpaws develops and persists at least 4 weeks(114, 115). In each experiment, mice were randomized into groups injected with either normal saline or acidified saline. At the time of injection, mice were briefly anesthetized using isoflurane, a hind leg cleaned using alcohol, and 20 µL of normal or acidified saline injected into the gastrocnemius muscle using a 31g needle. All animals were immediately returned to their home cage and observed by the investigator until fully ambulatory.

Mechanical Hypersensitivity Testing

The von Frey filament test is used to measure sensitivity to a non-noxious punctate pressure stimulus. All habituation and testing took place at light onset and was completed during the first 2 h of the light period. Mice were habituated to a galvanized steel mesh testing platform for a minimum of 60 min for 3 days prior to baseline testing. On testing days, mice were given a minimum of 30 min (or until quiet) to habituate to the testing platform. Calibrated filaments (0.07, 0.45, and 1.45 g pressure deflection) were presented in ascending order to the

glabrous skin of the hindpaw until they bowed slightly(117). Hindpaws were alternated until a total of 5 presentations per filament per paw was reached. Testing continued until all 3 filaments had been presented with a minimum of 1-min break between filament presentations. If mice became active, testing was suspended until mice were quiet before continuing. Positive responses were recorded when mice retracted the paw in response to the filament.

Experimental Design and Clinical Health Monitoring

A total of 30 C57B/L6 mice were used in this study. A subset (n = 14) of mice was implanted with telemetry units to record EEG and body temperature, which were used to determine sleep state (see later). Surgically implanted mice were given three weeks of recovery before the study began. All mice, irrespective of whether they were surgically implanted or uninstrumented, were 9-12 weeks at the time von Frey testing began. All mice underwent 3 days of baseline von Frey testing to determine mechanical sensitivity. For mice implanted with telemetry units, 2 days of baseline EEG and body temperature recordings were collected prior to sensitization injections. All mice were twice injected with either normal (n = 14 total; n = 6 instrumented) or acidified saline (n = 16 total; n = 8 instrumented), 5 days apart as described above. Mechanical sensitivity was assessed at baseline (before any injections) and 1, 3, 7, 14, 21 days after the second sensitization injection. EEG and body temperature were recorded from mice instrumented with telemeters for the duration of the protocol.

Daily food consumption, water consumption, and body weight were recorded at light onset throughout the experimental protocol. These measures

provided an assessment of the impact of musculoskeletal sensitization on the overall health of the animal.

Surgical Procedures

Mice that were implanted with the telemeters were deeply anesthetized with isoflurane (4% induction, 2% maintenance) and surgically implanted with telemeters (ETA10-F20, Data Sciences International, Minneapolis, MN) to permit monitoring of the electroencephalogram (EEG), core body temperature (CBT) and activity as previously reported(30, 204). Transmitter leads were passed subcutaneously to the base of the skull and attached to stainless steel screws (#80 × 1/8 in., Small Parts, Miami Lakes, FL) placed bilaterally over frontal and parietal cortices. These screws served as EEG recording electrodes. Mice were injected subcutaneously with Penicillin G Procaine (0.1 to 0.2 mL, 300,000 units/mL) immediately after surgery to reduce risk of infection. Perioperative pain management consisted of providing ibuprofen (0.2 mg/mL) in drinking water for 48 h after surgery and administration of buprenorphine (0.05 mg/kg, subcutaneously) at the time of surgery and for 2 days following surgery, if needed. Lidocaine and triple antibiotic ointment were applied topically at the incision site immediately after surgery. Mice were monitored during recovery from anesthesia until ambulatory and were then transferred to recording cages for recovery and habituation.

Physiological Monitoring and Data Acquisition

Signals from telemeters were fed to an analog converter (DSI ART Analog-8 CM) that converted EEG and temperature signals to voltages using a

transmitter-specific calibration factor provided by DSI. The output from the converter was captured by an AD board (model PCI-3033E, National Instruments) that re-digitized the data at 128 Hz with 16-bit precision. Temperature voltages were converted to engineering units by regression using calibration coefficients specific for each transmitter. General cage activity was detected using infrared sensors. All signals (EEG, core body temperature, and cage activity) were stored as binary files until further processing.

During acquisition, the EEG was digitally filtered using Chebyschev filters with 3^{rd} order coefficients into delta (0.5 - 4.5 Hz) and theta (6.0 - 9.0) Hz frequency bands. These filtered EEG signals were integrated over 1-s periods and stored as part of the binary file structure. Arousal state designations were made on the basis of visual inspection of the recordings using custom software (ICELUS, M. Opp, University of Michigan) written in LabView for Windows (National Instruments) as previously described(30, 204). Briefly, wakefulness (W), NREM (NREM) sleep, or REM (REM) sleep was determined for each 10-s epoch of the recording period based on the EEG, integrated delta and theta frequency components of the EEG, and general cage activity. Any epoch containing either movement artifacts or electrical noise was tagged and excluded from subsequent spectral analyses. The raw, non-integrated EEG signals were processed offline using fast Fourier transforms (FFT) to yield power spectra between 0.5 and 40 Hz in 0.5 Hz frequency bins. These spectra were computed by averaging the 5 consecutive 2-s EEG segments comprising each 10-s epoch. The resulting spectrum was matched to state to provide state-specific spectra.

Spectra were normalized as a percentage of total power across all frequencies for specific behavioral states within the 12-h light or dark period.

The extent to which sleep was consolidated or fragmented was determined by evaluating the number of transitions from one arousal state to the next. These determinations were made irrespective of arousal state designation and without the use of arbitrary criteria for sleep architecture parameters. Latency to REM sleep was defined and recorded as the time in minutes from light onset to the first REM sleep bout consisting of a minimum of 2 consecutive epochs (20 s) of REM sleep.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows. All data are presented as mean ± standard error of the mean (SEM). To determine the impact of manipulations across time, analyses were restricted to within-group (normal saline, acidified saline) comparisons for time spent in each behavioral state, core body temperature, food and water consumption, and body weights. These within-group comparisons were made by means of a general linear model for repeated measures using a within subjects factor of time. To determine if there was an effect of intramuscular injections on these parameters, a general linear model for repeated measures with between-subjects factor of treatment (normal saline, acidified saline) was used.

To determine if there was an effect of intramuscular injections on mechanical sensitivity, comparisons were made by evaluating the response incidence ([response per filament/5 possible responses] × 100) data for each

paw (ipsilateral, contralateral to intramuscular injection) from each individual monofilament, as well as a total response incidence ([Total responses per paw/15 possible responses] × 100). Repeated-measures ANOVA with between-subjects factor of treatment (control, acidified) and a within-subjects factor of time (day of experimental protocol) was used to test ipsilateral and contralateral paw data. An α level of $P \leq 0.05$ was accepted for all statistical tests as indicating significant departures between the groups across the testing period.

RESULTS

Bilateral Mechanical Hypersensitivity

Unilateral injections of acidified saline spaced 5 days apart produced mechanical hypersensitivity at the hindpaws relative to control animals injected with normal saline (Fig. 1). This hypersensitivity was detected across all 3-filament pressures and manifest as a significant increase in response incidence to von Frey testing that was apparent on day 3 and continued across all 21-protocol days. Total responsiveness to filaments of mechanically sensitized animals was significantly increased for the 3-week testing period when compared with control mice (between groups) and pre-injection baseline (within groups).

Sleep State Transitions

The number of state transitions during the baseline recording period did not significantly differ among mice subsequently randomized into the two treatment groups (Table 1). Within subjects analysis revealed modest, yet statistically significant increases in the number of state transitions for control mice only during the light period across all recording days. However, mice

injected with acidified saline and subsequently sensitized exhibited an increase in the number of state transitions during both the light and dark period post-injection. Furthermore, between subjects analysis revealed that experimental mice with musculoskeletal sensitization manifest a greater number of state transitions than control mice at all time points assessed during the protocol (Table 1).

Sleep Duration

The amount of NREM and REM sleep during the baseline recording period did not differ among mice subsequently randomized into the 2 treatment groups (Table 1). Within subjects analyses did not reveal a significant change in either NREM or REM sleep duration during the recording period for either injection group (Table 1). Similarly, between subjects analyses did not reveal a significant impact of musculoskeletal sensitization on NREM or REM sleep time (Table 1). Latency to REM sleep increased significantly for mice injected with acidified saline. The average REM sleep latency increased from 21 min at baseline to 75 min after musculoskeletal sensitization.

NREM Delta Power and Spectral Analysis

EEG spectral characteristics were analyzed from recordings obtained at baseline and days 2, 8, 15, and 20 after the second intramuscular injection. Delta power during NREM is a common measure of sleep intensity(205, 206), with NREM delta power increasing during recovery sleep after periods of prolonged wakefulness(24). Because of inter-animal variations in the EEG, all analyses were performed on values normalized relative to the 12 hour average NREM

delta power for the light and dark period ([Hourly value / 12h period average]*100)(23). At pre-injection baseline, normalized delta power during NREM sleep during the 12-h light or dark period did not significantly differ between mice subsequently randomized to injection groups. Within subjects analysis did not reveal a significant change in NREM delta power within injection groups across the recording period. Similarly, between subjects analysis did not reveal significant effects of musculoskeletal sensitization on normalized NREM delta power (Table 1).

State-specific EEG power spectra were normalized as a percentage of total power across all frequencies for specific behavioral states within the 12-h light or dark period. Statistical analyses were performed on bins in the delta (0.5 - 4.5 Hz) and theta (6.0 - 9.0 Hz) frequency bands for NREMS and REMS, respectively. Although statistical significance was not achieved across the frequency bands, there was a significant increase in the peak theta frequency of acidified saline injected mice during dark period REM sleep (Fig. 3).

Food Consumption, Water Consumption, Body Weight, and Core Body Temperature

Daily food consumption, water consumption, and body weight were not significantly impacted by intramuscular acidified saline injections (data not shown). Repeated-measures analysis did not reveal a significant effect of manipulation (normal saline, acidified saline) on these parameters.

Pre-injection baseline core body temperature did not differ among mice that were subsequently randomized to the injection groups. No significant effect

of injection was detected by repeated measures analysis within subjects or between injection groups (Table 1).

DISCUSSION

Approximately 20% of Americans report that pain or physical discomfort disrupts their sleep at least a few nights a week(207), and patients with chronic pain conditions often report sleep disruption as a comorbidity to their pain(157, 208). Although three of the most prevalent types of chronic pain in the United States are musculoskeletal; low back pain, neck pain, and facial pain(4), most preclinical studies of pain have focused on neuropathic or inflammatory pain(158, 159). Improving sleep can reduce next day pain(193), especially in patients with ongoing musculoskeletal pain(175). Persons with musculoskeletal disorders, including pain, have a lower quality of life as compared with other chronic ongoing health conditions(102). Sleep affects a wide range of homeostatic biological functions such as mood regulation, cardiovascular function, and cognitive functions including decision making, memory, and attention(1). The negative impact of musculoskeletal pain on sleep may in turn influence the collective well-being of the patient more than a chronic pain state independent of sleep disruption.

The novel finding of this study is that musculoskeletal sensitization fragments sleep of mice without altering the total amount of time spent in NREM sleep, REM sleep, or wakefulness. Furthermore, musculoskeletal sensitization does not impact the clinical health of mice as evidenced by measures of body weight, food and water consumption and body temperature. Our observations

that acidified saline injections into mice induce bilateral secondary mechanical hypersensitivity replicate findings in the literature(114, 115), and suggest this model may be of utility for studies of interactions between sleep and musculoskeletal sensitization.

In this present study, musculoskeletal sensitization did not alter the amount of time spent in NREM or REM sleep. The literature is varied with respect to the extent to which sleep amounts are disrupted during chronic pain(161, 164). For example, chronic constriction injury (CCI), in which a surgically implanted suture constricts the sciatic nerve and produces allodynia at the hindpaw, in one study is reported to transiently alter sleep of rats(161), an effect that was most robust during the first 10 days after nerve constriction. Another study using the same model in rats reported no changes to sleep(164). Differences in findings between these studies may be due to the post-injury time course selected for recording. In the first study sleep state was monitored continuously 21 days after surgery(161), whereas the study that saw no change recorded for single days with the first occurring 13 days post-surgery(164). This difference in time course suggests that CCI may have resulted in significant changes in sleep during the first 10 days post-surgery as previously reported, but beginning recordings on day 13 may have missed this significant change.

It is also possible that subpopulations of rodents differ in their susceptibility to chronic pain(166, 209, 210). Monassi and colleagues identify 3 distinct phenotypes of responders after CCI; animals that manifest *pain with persistent disability*, those that exhibit *pain with only transient disability*, and

those that indicate *pain, but no disability*(166). In these studies, all rats developed sensitivity to mechanical and cold stimuli to the same degree, but exhibited different phenotypic changes in sleep. Rats exhibiting *pain with persistent disability* spent less time in NREM sleep and increased wakefulness during both the light and dark periods, an effect that persisted for the 8-day follow up period after CCI. Rats with *pain and only transient disability* spent less time in NREM sleep and increased wakefulness, but only during the light period, and this effect normalized by the end of the 8-day recording period. Sleep was not altered in rats that exhibited *pain without disability*(166).

To investigate a role for astrocytes as mediators of pain and disability after CCI, the periaqueductal gray (PAG) was stained for glial fibrillary acid protein (GFAP), a marker of activated astrocytes. Increased staining for GFAP was detected in the lateral and caudal ventrolateral columns of the PAG in rats exhibiting *pain with persistent disability*(210). The anatomical specification of this upregulation of GFAP suggests that afferents from both the spinal column and nucleus of the solitary tract may be critical as the ventrolateral PAG is the site of termination. Furthermore, mRNA expression for markers of cell death in the PAG is upregulated in rats with *pain and persistent disability*(209). Because the PAG is a brain region involved in the regulation of sleep(107, 211, 212) and pain(108, 211), data from these collective studies indicate that the PAG may serve as a critical site of integration for interactions between pain and sleep.

Although the PAG may be functionally implicated in regulating sleep and pain, the PAG has limited direct projections to the spinal cord(213). The PAG

does however, have direct projections to the rostral ventral medulla (RVM), which in turn projects to the spinal cord(214). The RVM is involved in pain transmission(108, 214, 215) and is implicated in mediating muscle sensitivity(119, 216, 217). Microinjections of local anesthetic(119) or NMDA receptor antagonists(217) into the RVM after bilateral mechanical hypersensitivity has developed reverses mechanical hypersensitivity. After one intramuscular injection with acidified saline, glycine concentrations in the RVM are reduced(216). Following the second acidified saline injection, but not the first, glutamate concentration increase in the RVM(216). The RVM contributes to the maintenance of hypersensitivity in the musculoskeletal sensitization model through regulation of neurotransmitter release, changes in NMDA receptor expression, and changes in neuronal excitability(110, 216, 217). As such, data support the hypothesis that the RVM and PAG may independently or synergistically contribute to the sleep fragmentation and mechanical hypersensitivity associated with musculoskeletal sensitization. Future experiments will test this mechanistic hypothesis.

Sleep fragmentation, characterized by an increased number of transitions between arousal states, is frequently reported in preclinical models of chronic pain. Several studies report changes in sleep of rats using an adjuvant-induced arthritis model(165, 197). Sleep of arthritic rats is characterized by increased total number of sleep and wakefulness bouts, increased microarousals, decreased NREM and REM sleep duration, and a reduction in sleep efficiency(165, 197). Sleep fragmentation has also been recorded in both male and female rats with

experimental osteoarthritis characterized by reduced NREM and REM sleep and reduced sleep efficiency(198). Arthritis induced by intra-articular knee injections of uric acid produces lasting increases in wakefulness, reductions in REM sleep, and REM bout numbers of rats(163). Sleep is also fragmented during orofacial pain, a model in which chronic pain is induced by injecting Freund's adjuvant into the masseter muscle. Under these conditions, sleep efficiency is reduced and the amount of time spent in wakefulness is increased(218, 219). In mice with experimental neuropathic pain induced by sciatic nerve ligation, NREM sleep is suppressed and wakefulness increased for at least 28 days following surgery(200). The common thread among these studies of chronic pain using different preclinical models is one of fragmented sleep, usually accompanied by a change in sleep duration. Our findings of increased state transitions during musculoskeletal sensitization are consistent with these previous observations and contribute to the growing literature of the manner in which sleep is disrupted during chronic pain.

Fibromyalgia is a chronic condition of unknown etiology characterized by widespread musculoskeletal pain and sleep disruption(220). Among chronic pain conditions, fibromyalgia is unique because un-refreshing sleep is a diagnostic factor(221, 222). Patients often complain of non-restorative sleep, insomnia, early morning awakenings, and overall poor sleep quality(223-225). The pain that is experienced by fibromyalgia patients correlates with quality of sleep, such that diminished subjective sleep quality is associated with enhanced pain(226, 227). Changes in the EEG of patients with fibromyalgia are characterized by an

intrusion of alpha waves into the NREM sleep that corresponds with next day pain(224, 228), although recent studies do not replicate these findings(229). Our study demonstrates changes in theta frequency components of the EEG spectra during REM sleep that persist for at least 20 days post sensitization. At present, the functional significance of altered EEG spectra during musculoskeletal sensitization in this model remains to be determined.

The economic costs and personal impact of chronic pain and sleep disruption on quality of life underscore the need for additional treatment options. Clinical surveys identify that subjectively restorative sleep reduces next day pain, especially in patients with musculoskeletal pain(175). Conversely, reduction of daytime pain does not predict subsequent restorative sleep(175), and a lack of restorative sleep could further exacerbate pain. These relationships between sleep and pain suggest a “vicious cycle” that perhaps may be broken by focusing on manipulation of sleep, not pain, as a critical target for intervention. In patients with chronic pain and sleep disturbance, it may be possible to alleviate or reduce pain by effective interventions to improve sleep quality using targeted pharmacological treatments, behavioral treatments, or a combined approach. Indeed, recent studies demonstrate that cognitive behavioral therapy to treat insomnia in patients with fibromyalgia and other chronic pain conditions also is effective in reducing pain(230, 231).

Our data demonstrate that musculoskeletal sensitization using acidified saline injections fragments sleep of mice without reducing amounts of NREM or REM sleep. Food and water intake, as well as body weight, are not altered during

musculoskeletal sensitization in this model. Collectively, our data support findings in the clinical literature that musculoskeletal pain fragments sleep. Our present results are an initial attempt to determine the extent to which sleep is disrupted during musculoskeletal sensitization. These results demonstrate a relationship between musculoskeletal sensitization and sleep, yet do not provide knowledge about mechanisms underlying these interactions. The similarity between the patient reported experience of sleep disruption during musculoskeletal pain and sleep fragmentation of mice during musculoskeletal sensitization provides a framework to begin investigating the mechanisms underlying relationships between musculoskeletal sensitivity and sleep.

FIGURES

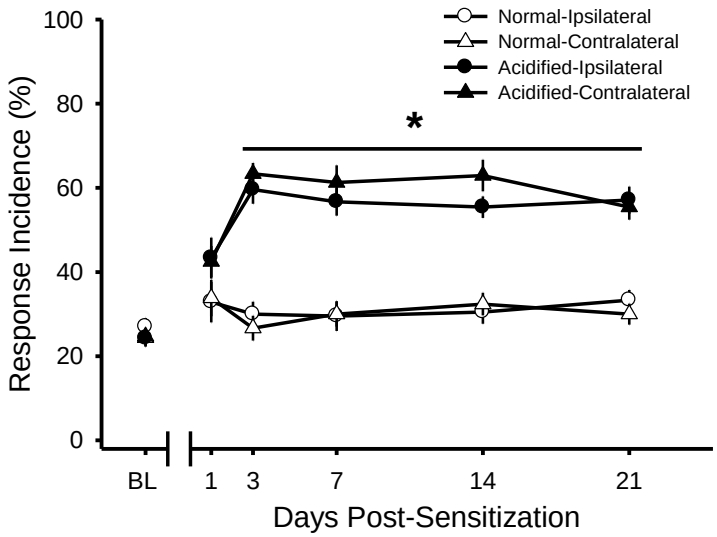

Figure 1. Musculoskeletal sensitization enhances bilateral responses to von Frey testing.

Mice injected with acidified saline (n = 16) exhibit mechanical hypersensitivity for at least 21 days, whereas mechanical hypersensitivity does not develop in mice injected with normal pH saline (n = 14). Responsiveness to von Frey filaments are plotted as mean ± SEM total response incidence percent ([total responses / total filament presentations] × 100) per paw. * $p \leq 0.05$ vs. normal pH saline injection.

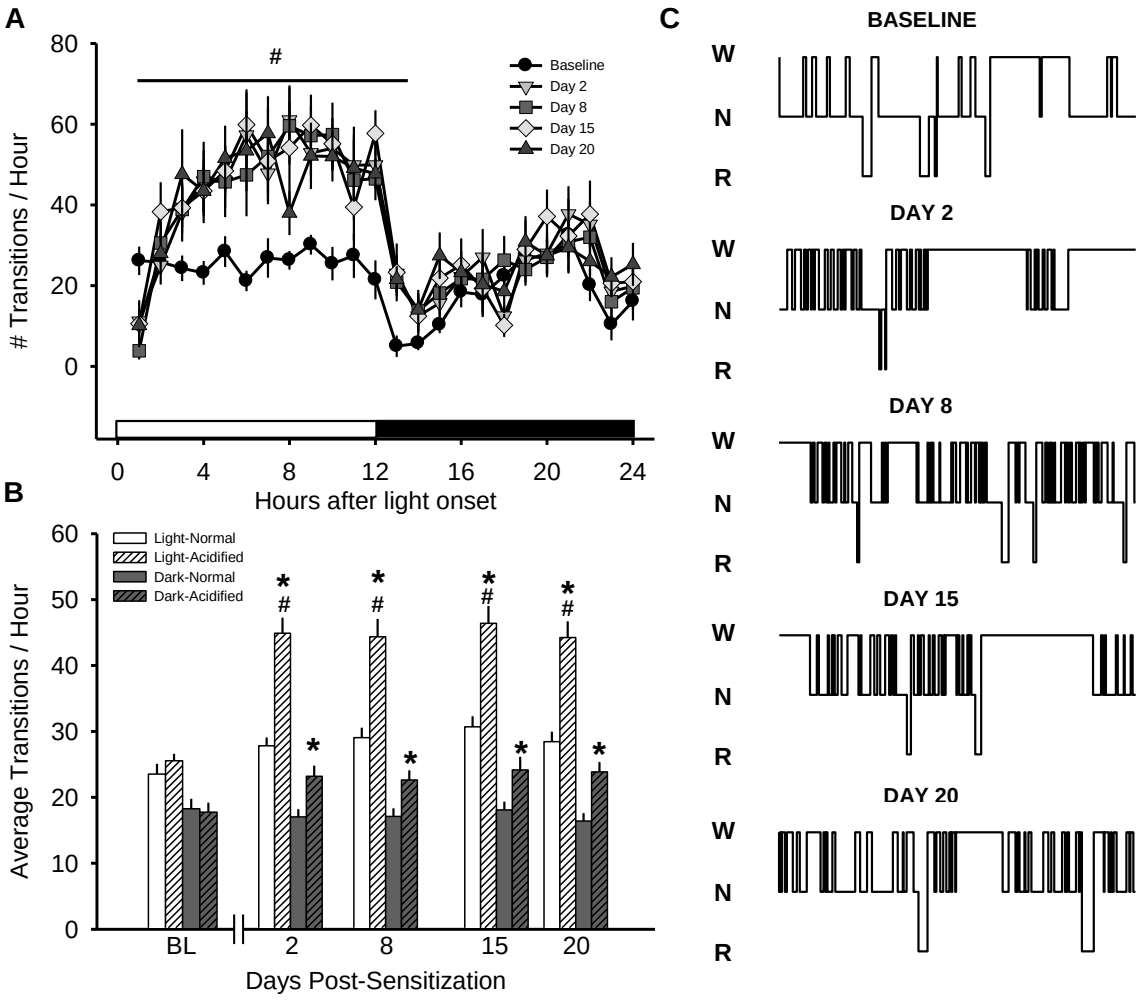

Figure 2. Sleep is fragmented after musculoskeletal sensitization with acidified saline.

The total number of transitions/h is plotted across the 24-h light/dark period only for animals injected with acidified saline (n = 8). Symbols are the mean ± SEM for pre-injection baseline and for 20 days after mechanical hypersensitivity is induced. Acidified saline injections fragment of sleep during the light period. (B) The average number of transitions/h during the 12-h light or dark period is plotted for pre-injection baseline (BL), and for days 2, 8, 15, and 20 after mechanical hypersensitivity is induced. Values are the mean ± SEM for n = 8 mice. (C) Representative hypnograms from one mouse obtained during pre-injection baseline, and at days 2, 8, 15, and 20 after induction of mechanical hypersensitivity. Hypnograms are from a 1-h recording 10 h after light onset during each of the days depicted. W-Wakefulness, N-NREM, R-REM sleep # $p \leq 0.05$ vs. pre-injection baseline; * $p \leq 0.05$ vs. normal pH saline injection.

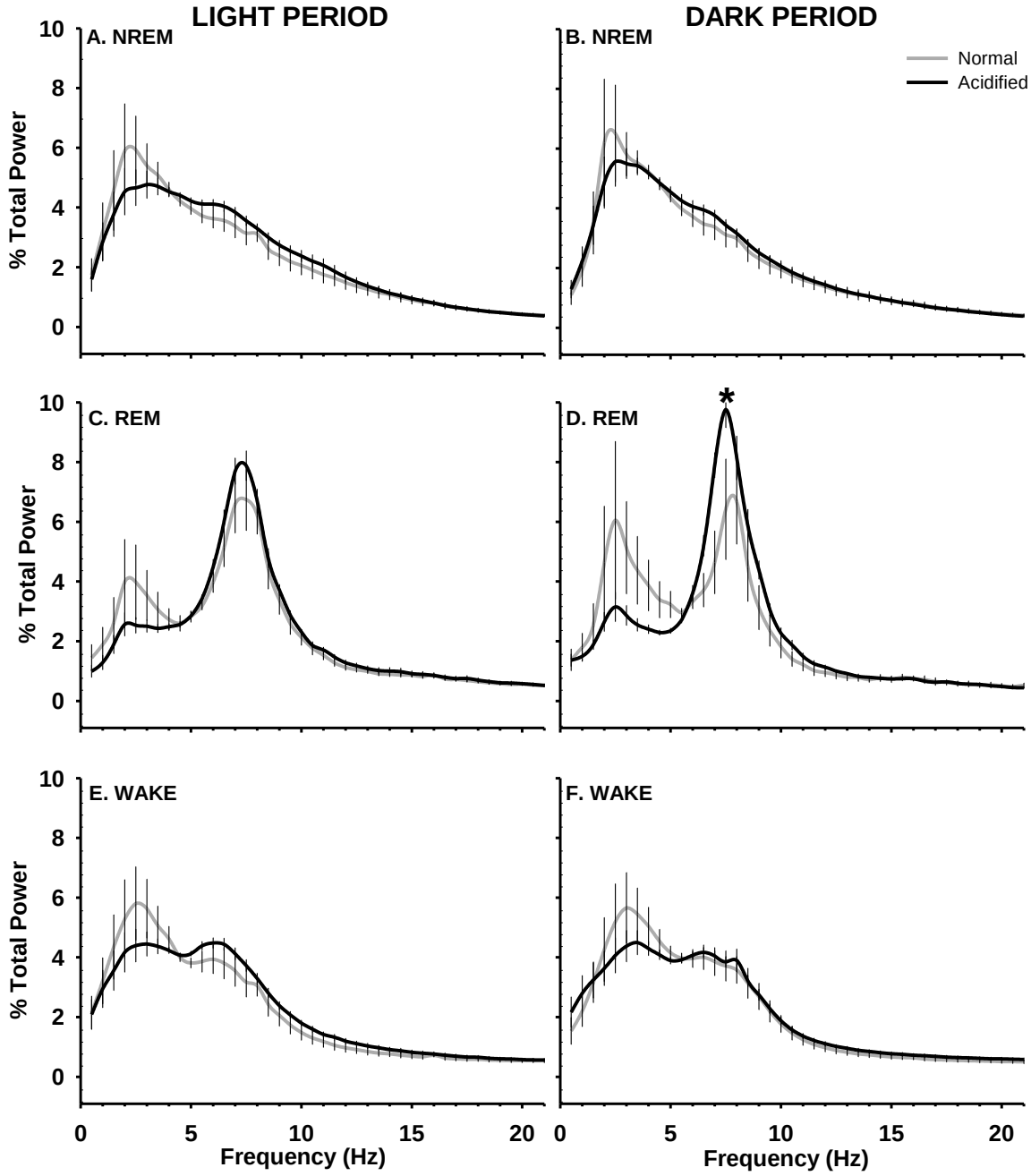
LIGHT PERIOD
DARK PERIOD
A. NREM
B. NREM
Normal
Acidified
C. REM
D. REM
*
E. WAKE
F. WAKE
% Total Power
Frequency (Hz)
0
2
4
6
8
10
0
5
10
15
20

Figure 3. State-specific electroencephalogram (EEG) power spectra are altered during musculoskeletal hypersensitivity.

State-specific EEG power spectra were obtained from mice injected with either normal pH saline (n = 6; thin grey lines) or acidified pH saline (n = 8; thick black lines). Data presented were obtained 20 days following musculoskeletal sensitization (or control injections). Spectra were normalized as a percentage of total power within each frequency band during the 12-h light or dark period and are plotted as mean ± SEM for each frequency bin. Statistical analyses were performed on bins comprising the delta frequency band (0.5 - 4.5 Hz) and the theta frequency band (6.0-9.0 Hz) for NREMS and REMS, respectively. A significant change was detected between the peak theta frequency during the dark period for NREM sleep between injection groups on day 20. * $p \leq 0.05$ vs. normal pH saline injection.

BASELINE		DAY 2		DAY 8		DAY 15		DAY 20	
LIGHT	DARK	LIGHT	DARK	LIGHT	DARK	LIGHT	DARK	LIGHT	DARK
NREM (% recording time)									
52.5 ± 2.5	33.0 ± 2.3	51.8 ± 1.9	32.3 ± 2.2	53.4 ± 2.0	28.5 ± 1.8	53.7 ± 2.0	31.8 ± 2.0	55.8 ± 2.0	31.1 ± 2.0
57.2 ± 1.7	31.3 ± 2.5	49.2 ± 1.8	32.6 ± 2.1	48.8 ± 2.2	31.8 ± 2.0	50.6 ± 2.0	31.0 ± 1.9	50.6 ± 2.0	34.2 ± 1.8
REM (% recording time)									
5.1 ± 0.3	3.3 ± 0.4	5.2 ± 0.3	2.1 ± 0.3	5.2 ± 0.4	2.1 ± 0.3	5.2 ± 0.3	2.0 ± 0.3	5.6 ± 0.3	1.7 ± 0.3
3.9 ± 0.3	2.3 ± 0.1	5.0 ± 0.4	2.0 ± 0.4	5.1 ± 0.4	1.8 ± 0.3	4.7 ± 0.4	1.7 ± 0.3	5.7 ± 0.4	1.7 ± 0.3
WAKEFULNESS (% recording time)									
42.4 ± 2.8	63.6 ± 2.7	43.0 ± 2.2	65.5 ± 2.4	42.8 ± 1.8	70.0 ± 2.1	41.1 ± 2.2	66.2 ± 2.2	38.6 ± 2.2	67.1 ± 2.2
38.9 ± 1.9	66.4 ± 2.7	45.8 ± 2.0	65.4 ± 2.4	46.1 ± 2.4	66.4 ± 2.2	44.7 ± 2.1	67.3 ± 2.1	43.6 ± 2.2	64.1 ± 2.0
T CORE (˚C)									
36.5 ± 0.11	37.1 ± 0.04	36.0 ± 0.06	37.1 ± 0.10	36.2 ± 0.08	37.1 ± 0.10	35.8 ± 0.08	37.0 ± 0.07	35.7 ± 0.09	37.1 ± 0.07
35.9 ± 0.10	37.1 ± 0.06	36.3 ± 0.09	37.1 ± 0.10	36.0 ± 0.10	37.2 ± 0.06	35.8 ± 0.09	37.0 ± 0.07	35.9 ± 0.09	37.1 ± 0.05
DELTA PWR (arbitrary unit)									
.87813 ± 0.031	1.1358 ± 0.025	.90411 ± 0.019	1.108 ± 0.023	0.8884 ± 0.023	1.120 ± 0.025	0.8958 ± 0.028	1.113 ± 0.023	0.9506 ± 0.029	1.051 ± 0.027
.90586 ± 0.011	1.1130 ± 0.016	.94988 ± 0.017	1.053 ± 0.015	0.9560 ± 0.017	1.045 ± 0.015	.9396 ± 0.016	1.063 ± 0.016	0.9534 ± 0.017	1.0433 ± 0.014
TRANSITIONS (average # per hour)									
23.5 ± 1.5	18.3 ± 1.5	27.8 ± 1.3 #	17.0 ± 1.2	29.1 ± 1.5 #	17.1 ± 1.2	30.7 ± 1.6 #	18.1 ± 1.3	28.5 ± 1.5 #	16.4 ± 1.2
25.5 ± 1.0	17.8 ± 1.4	44.9 ± 2.4 *#	23.2 ± 1.6 *#	44.3 ± 2.7 *#	22.6 ± 1.5 *#	46.4 ± 2.6 *#	24.2 ± 2.0 *#	44.2 ± 2.5 *#	23.9 ± 1.5 *#
					Normal				
					Acidified				

Table 1. Sleep duration, core temperature, normalized delta power, and sleep state transitions across the recording period.

Sleep duration, core body temperature, normalized delta power, and arousal state transitions averaged during the light and dark period. Normal (n=6) and acidified (n=8). NREM, REM and Wakefulness are presented as a percentage of total recording time for the light or dark period respectively. Core body temperature is presented in ˚C. Delta power is normalized relative to the 12h average per mouse. State transitions are presented as the average number of state transitions per hour during the light or dark period. T Core- Core body temperature. #, $p \leq 0.05$ vs. pre-injection baseline; *, $p \leq 0.05$ vs. normal pH saline injection.

CHAPTER III
SLEEP FRAGMENTATION EXACERBATES MECHANICAL HYPERSENSITIVITY AND ALTERS SUBSEQUENT SLEEP-WAKE BEHAVIOR IN A MOUSE MODEL OF MUSCULOSKELETAL SENSITIZATION[2]

ABSTRACT

Study Objectives: Sleep deprivation or sleep disruption enhances pain in human subjects. Chronic musculoskeletal pain is prevalent in our society, and constitutes a tremendous public health burden. Although pre-clinical models of neuropathic and inflammatory pain demonstrate effects on sleep, few studies focus on musculoskeletal pain. We previously reported that musculoskeletal sensitization alters sleep of mice. In this study we hypothesize that sleep fragmentation during the development of musculoskeletal sensitization will exacerbate subsequent pain responses and alter sleep-wake behavior of mice.

Design: This is a pre-clinical study using C57BL/6J mice to determine the impact of sleep fragmentation combined with musculoskeletal sensitization on behavioral outcomes.

Methods: Musculoskeletal sensitization, a model of chronic muscle pain, was induced using two unilateral injections spaced five days apart of acidified

[2] The work presented in this chapter are under review for publication: Sutton, B.C. & Opp, M.R. Sleep fragmentation exacerbates mechanical hypersensitivity and alters subsequent sleep-wake behavior in a mouse model of musculoskeletal sensitization. SLEEP.

saline (pH 4.0) into the gastrocnemius muscle. Musculoskeletal sensitization manifests as mechanical hypersensitivity as determined by von Frey filament testing at the hindpaws. Sleep fragmentation took place during the consecutive 12h light periods of the 5 days between intramuscular injections. EEG and body temperature were recorded from some mice at baseline and for three weeks after musculoskeletal sensitization. Mechanical hypersensitivity was determined at pre-injection baseline and on days 1, 3, 7, 14, and 21 after sensitization. Two additional experiments were conducted to determine the independent effects of sleep fragmentation and musculoskeletal sensitization on mechanical hypersensitivity.

Results: Five days of sleep fragmentation by itself did not induce mechanical hypersensitivity, whereas sleep fragmentation combined with musculoskeletal sensitization resulted in prolonged and exacerbated mechanical hypersensitivity responses. Sleep fragmentation combined with musculoskeletal sensitization had an impact on subsequent sleep of mice as demonstrated by increased sleep-wake state transitions during the light and dark periods; changes in NREM sleep, REM sleep and wakefulness; and altered delta power during NREM sleep. These effects persisted for at least three weeks post-sensitization.

Conclusions: Our data demonstrate that sleep fragmentation combined with musculoskeletal sensitization exacerbates the physiological and behavioral responses of mice to musculoskeletal sensitization, including mechanical hypersensitivity and sleep. These data contribute to a growing literature demonstrating bi-directional relationships between sleep and pain. The

prevalence and incidence of insufficient sleep and pathologies characterized by chronic musculoskeletal pain are increasing in the United States. These demographic data underscore the need for research focused on insufficient sleep and chronic pain so that the quality of life for the millions of individuals suffering these conditions may be improved.

INTRODUCTION

Sleep loss negatively impacts homeostatic functions including metabolism, cognition, emotional regulation, immune function, cardiovascular function, and pain(1, 9, 176, 232-234). In the United States nearly 30% of the adult population reports insufficient sleep(207) and roughly 70 million Americans have a diagnosed sleep disorder(207). Sleep disorders, including insomnia, narcolepsy, and sleep apnea, fragment or restrict sleep(235-237). Experimental deprivation or restriction of sleep in humans and rodents enhances pain(171, 173, 180, 183, 238, 239). Subjectively sleepy persons have reduced pain thresholds compared to well-rested individuals(192), and chronic pain is the most frequent co-morbidity associated with primary insomnia(169). Epidemiological studies identify subjectively poor sleep quality as an independent risk factor for the development of chronic pain conditions, especially those characterized by musculoskeletal pain(55, 194).

Musculoskeletal pain is prevalent in our society, with low back pain, neck pain, and facial pain(4) constituting a major public health burden. Arthritis (primarily rheumatoid), spinal pain (including low back pain), and fibromyalgia, all musculoskeletal, are the most prevalent chronic pain conditions associated with

insomnia(194-196). Data derived from pre-clinical studies of rodents support clinical findings that sleep loss reduces pain threshold(180, 182, 183). In a rodent model of musculoskeletal pain, musculoskeletal sensitization induces long-lasting mechanical hypersensitivity characterized by increased responsiveness to mechanical stimuli(114-116). We recently reported that sleep of mice in which musculoskeletal sensitization has been induced is fragmented(116). In this study, we hypothesize that sleep fragmentation during the period when musculoskeletal sensitization develops will exacerbate the effects of musculoskeletal sensitization. To test this hypothesis, we fragmented sleep of mice, induced musculoskeletal sensitization, and determined the impact on subsequent sleep-wake behavior and mechanical hypersensitivity. We now report that sleep fragmentation combined with musculoskeletal sensitization exacerbates for prolonged periods mechanical hypersensitivity and alters multiple facets of mouse sleep.

METHODS

Animals

Adult male C57BL/6J mice (4-6 weeks of age; 25 g) were used in this study. All mice were purchased from Jackson Laboratory (Bar Harbor, ME), and maintained on a 12:12 h light:dark cycle at 27 °C with ad libitum access to food and water. All procedures using mice in these studies were approved in advance by the University of Washington Institutional Animal Care and Use Committee (IACUC), in accordance with the US Department of Agriculture Animal Welfare

Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals.

Clinical health of mice was monitored daily at light onset and consisted of measures of food consumption, water consumption, and body weight. These data were collected throughout the surgical recovery period and for the duration of the protocols.

Musculoskeletal Sensitization

Musculoskeletal sensitization was induced by two unilateral injections of acidified saline into the gastrocnemius muscle. The injections were spaced five days apart and consisted of either normal (pH 7.2; control) or acidified (pH 4.0) saline. Acidified saline injections in this protocol producee a robust bi-lateral secondary mechanical hypersensitivity at the hindpaws that lasts at least 4 weeks(114-116). At the time of injection, mice were briefly anesthetized using isoflurane, a hind leg cleaned using alcohol, and 20 µL of normal or acidified saline injected into the gastrocnemius muscle using a 31g needle. All animals were immediately returned to their home cage and observed by the investigator until fully ambulatory.

Mechanical Hypersensitivity Testing

The von Frey filament test measures sensitivity to a non-noxious punctate pressure stimulus using calibrated monofilaments. Determination of mechanical hypersensitivity was done as previously reported(116). Briefly, mice were habituated to the testing procedure and the galvanized steel mesh testing platform for a minimum of 60 minutes each day for 3 days prior to obtaining

baseline values. On testing days, mice were placed on the testing platform for a minimum of 30 minutes (or until quiet). Calibrated filaments (0.07, 0.45, & 1.45 g pressure deflection) were then presented in ascending order to the glabrous skin of the hindpaws until they bowed slightly(116, 117). Hindpaws were alternated until a total of 5 presentations per filament per paw were reached. Each of the 3 filaments were presented with a minimum 1-minute break between filament presentations. Positive responses were recorded when mice retracted the paw in response to the filament pressure. If mice became active, testing was suspended until they were quiet. All testing was done during the first 2 hours of the light period.

Sleep Fragmentation

All animals undergoing sleep fragmentation were placed into the sleep disruption devices one day prior to the start of the sleep fragmentation protocol. The sleep disruption device consists of a circular Plexiglas chamber divided to form two compartments(65). The floor of the chamber is a motorized disc that rotates for specific durations as selected by the investigator.

Individual animals were placed into separate compartments prior to device habituation. Device habituation consisted of rotation of the disc for 8 seconds once every 30 minutes during one 12h light period of the 12:12 light:dark cycle. No disc rotation occurred during the dark period, during which mice were left undisturbed. Intramuscular injections and the beginning of sleep fragmentation began at light onset the day after habituation. Sleep was fragmented for 5 days by disc rotations that lasted for 8 seconds and occurred every 30 seconds, on

average, during the 12h light periods. During the 12h dark periods, the disc did not rotate and mice were free to behave normally. The direction of disc rotation and exact inter-rotation interval were computer-randomized to prevent behavioral adaptation of the animals to the rotations. Each disc rotation was greater than 180 degrees to ensure the mouse had to move to avoid bumping into the center divider of the chamber. We have demonstrated that this method of fragmenting sleep of mice is effective in protocols lasting up to 9 days(65).

Surgical Procedures

Mice from which recordings of the electroencephalogram (EEG) were to be obtained were deeply anesthetized with isoflurane (4% induction, 2% maintenance) and surgically implanted with telemeters (ETA10-F20, Data Sciences International, Minneapolis, MN). As previously described(30, 116, 240), transmitters were implanted in the peritoneum and leads were passed subcutaneously to the skull and attached to stainless steel screws (#80 × 1/8 in., Small Parts, Miami Lakes, FL) placed bilaterally over frontal and parietal cortices. These screws served as EEG recording electrodes. Mice were injected subcutaneously with Penicillin G Procaine (0.1 to 0.2 mL, 300,000 units/mL) immediately after surgery to reduce risk of infection. Perioperative pain management consisted of ibuprofen in drinking water (0.2 mg/ml; beginning 24 h before surgery and continuing for 48 h after surgery) and administration of buprenorphine (0.05 mg/kg, subcutaneously) at the time of surgery and for two days following surgery, if needed. Lidocaine and triple antibiotic ointment were applied topically at the incision site immediately after surgery. Mice were

monitored until ambulatory and then transferred to recording cages for recovery and acclimation.

Data Acquisition

Signals from telemeters were fed to an analog converter (DSI ART Analog-8 CM) that converted EEG and temperature signals to voltages using transmitter-specific calibration factors provided by DSI. The output from the converter was captured by an AD board (model PCI-3033E, National Instruments) that re-digitized the data at 128 Hz with 16-bit precision. Temperature voltages were converted by regression using calibration coefficients specific for each transmitter. General activity in the cage was detected using infrared sensors (BioBserve, GmbH, Bonn, Germany). Movements detected by the sensors were converted to a voltage output, the magnitude of which was directly related to the magnitude of movements detected. All signals (EEG, core body temperature, and cage activity) were stored as binary files until further processing.

During acquisition, the EEG was digitally filtered using Chebyschev filters with 3^{rd} order coefficients into delta (0.5 - 4.5 Hz) and theta (6.0 - 9.0 Hz) frequency bands. These filtered EEG signals were integrated over 1-s periods and stored as part of the binary file structure. Arousal state designations were made with 10-s resolution on the basis of visual inspection of the recordings using custom software (ICELUS, M. Opp, University of Washington) written in LabView for Windows (National Instruments). Arousal state was assigned for each 10-s interval on the basis of the EEG, body movements, and integrated

delta and theta frequency values using previously published criteria(116, 204, 240). Any epoch during which the EEG contained either movement artifacts or electrical noise was tagged and excluded from subsequent spectral analyses. The raw, non-integrated EEG signals were processed offline using fast Fourier transforms (FFT) to yield power spectra between 0.5 and 40 Hz in 0.5 Hz frequency bins. These spectra were computed by averaging the five consecutive 2-s EEG segments comprising each 10-s epoch. The resulting spectrum was matched to state to provide state-specific spectra. Our primary focus in this study was power in the delta frequency band during NREM sleep. These values for delta power during NREM sleep were obtained by summing the values of all 0.5 Hz frequency bins from 0.5 - 4.5 Hz.

The extent to which spontaneous sleep was consolidated or disrupted was determined by evaluating the number of transitions from one arousal state to the next. These determinations were made as previously described(116, 204, 240) irrespective of arousal state designation and without the use of arbitrary criteria for sleep architecture parameters.

Determination of the impact of sleep fragmentation on mechanical hypersensitivity

Three groups of mice (n=58 total) were used in this study to determine the combined effects of sleep fragmentation and musculoskeletal sensitization on outcome measures of interest. Mice in group 1 (n=12) were used to determine the impact of 5 days of sleep fragmentation per se on mechanical hypersensitivity. These animals did not receive intramuscular injections. All

mice underwent 3 days of habituation to the von Frey testing platform and 3 days of baseline von Frey testing (described earlier). Mice were then habituated to the sleep disruption device, after which they were subjected to sleep fragmentation during the 12h light period for 5 consecutive days. After the 5-day sleep fragmentation protocol, mice were housed singly under standard conditions. Von Frey testing was performed at baseline (BL), on the 3rd day of sleep fragmentation, and days 1, 3, & 7 post-fragmentation.

Whereas mice in group 1 were used to determine the impact of sleep fragmentation on mechanical hypersensitivity, mice in group 2 (n=24) were used to determine the effect of musculoskeletal sensitization without sleep fragmentation on mechanical hypersensitivity. These mice were not instrumented, and were housed singly in standard caging throughout the experimental period. Mice underwent habituation and baseline von Frey testing to determine mechanical hypersensitivity prior to experimental manipulations. Mice were subsequently randomized to a musculoskeletal sensitization group [normal saline (control animals) or acidified saline; n=12 per injection group], with intramuscular injections administered 5 days apart. Mechanical hypersensitivity was assessed on days 1, 3, 7, 14, and 21 following sensitization.

We previously reported results of an experiment that demonstrated the impact of musculoskeletal sensitization on sleep of mice(116). Mice in that study were allowed spontaneous behavior during the sensitization period. The purpose of this present experiment was to determine the impact of sleep fragmentation during the musculoskeletal sensitization period on mechanical hypersensitivity

and subsequent sleep-wake behavior. Twenty two mice (group 3) were used in this experiment, a subset of which (n=16) was instrumented to allow determination of sleep-wake behavior (Figure 4). These n=16 mice were implanted with telemeters to record EEG and core body temperature as described earlier. After recovery, baseline recordings were obtained from mice implanted with telemetry units for 2 days prior to experimental manipulation. All mice were habituated and underwent baseline von Frey testing for mechanical hypersensitivity. For all mice used in this experiment (instrumented, un-instrumented), sleep fragmentation started after the first intramuscular injection at light onset and ended at light onset 5 days later when the second sensitization injection was given (Figure 4). All mice were removed from the sleep disruption device and returned to single housed standard caging at the end of the 5 day sleep fragmentation period. Mechanical hypersensitivity was measured on days 1, 3, 7, 14, and 21 post-sensitization. Recordings were obtained from mice instrumented with telemeters for 22-days after sensitization. Two instrumented mice were excluded from data analysis due to poor EEG signal quality, reducing the final sample size of instrumented mice to n=14 (7 mice per injection group).

Statistical Analysis

Statistical analyses were performed using SPSS for Windows. All data are presented as mean ± standard error of the mean (SEM). To determine the impact of manipulations across time, analyses were restricted to within-group (normal saline, acidified saline) comparisons for time spent in each behavioral state, core body temperature, and clinical data. These within-group comparisons

were made by means of a general linear model for repeated measures. To determine if there was an effect of intramuscular injections on sleep, core body temperature, and clinical data, repeated-measures ANOVA with between-subjects factor of treatment (normal saline, acidified saline) and a within-subjects factor of time (day of experimental protocol).

To determine if there was an effect of intramuscular injections on mechanical sensitivity, the response incidence ([response per filament / 5 possible responses] x 100) data for each paw (ipsilateral, contralateral) from each individual monofilament, as well as a total response incidence ([Total responses per paw/15 total possible responses] x 100) were analyzed. Repeated-measures ANOVA with between-subjects factor of treatment (normal saline, acidified saline) and a within-subjects factor of time (day of experimental protocol) was used to test ipsilateral and contralateral von Frey data. An alpha level of $p \leq 0.05$ was accepted for all statistical tests as indicating significant departures between the groups across the testing period.

RESULTS

Mechanical hypersensitivity

Data from mice in group 1 demonstrated that sleep fragmentation by itself for 5 days had no significant impact on mechanical hypersensitivity (Figure 5). As such, sleep fragmentation by this protocol did not independently induce mechanical hypersensitivity. As previously published, unilateral injections of acidified saline 5 days apart produced bilateral mechanical hypersensitivity at the hindpaws (group 2; Figure 6)(114-116). Mechanical hypersensitivity after

musculoskeletal sensitization lasted at least 21 days, which was the duration von Frey testing lasted in this study.

Impact of sleep fragmentation combined with musculoskeletal sensitization on mechanical hypersensitivity and subsequent sleep-wake behavior

Whereas control mice (normal saline injections) in group 3 that were subjected to sleep fragmentation did not develop mechanical hypersensitivity, mice in which musculoskeletal sensitization had been induced by injections of acidified saline exhibited mechanical hypersensitivity on the first post-sensitization day (Figure 7). Mechanical hypersensitivity in mice subjected to the combined manipulations of sleep fragmentation and musculoskeletal sensitization persisted for 21 post-sensitization (Figure 7). The observation that mechanical hypersensitivity was apparent on the first post-sensitization day in mice subjected to sleep fragmentation during the musculoskeletal sensitization period was unexpected. Our previous studies(116) and data obtained in this study from mice in group 2, demonstrated that mechanical hypersensitivity does not manifest in this model until the 3rd post-sensitization day. Because the only factor that differed in this experiment was sleep fragmentation during the sensitization period, we compared response incidence values obtained from mice in group 2 that had undisturbed sleep in their home cages during the sensitization period with those from mice in group 3 that had sleep fragmented during the sensitization period. Direct comparison of the impact of undisturbed sleep or fragmented sleep on mechanical hypersensitivity is presented in Figure

8. Response incidence values obtained from animals injected with normal saline did not differ at any time irrespective of whether or not they had been subjected to sleep fragmentation. Furthermore, response incidence values did not differ from pre-injection baseline values, indicating that being housed on the sleep disruption device and being subjected sleep fragmentation per se did not induce mechanical hypersensitivity. However, mice in which sleep was fragmented during the period of musculoskeletal sensitization developed mechanical hypersensitivity that was of greater magnitude than that of mice allowed undisturbed sleep (Figure 8). This increased mechanical hypersensitivity observed in mice subjected to the combination of sleep fragmentation and musculoskeletal sensitization was apparent on the first post-sensitization day and on post-sensitization day 21 (Figure 8).

In addition to its impact on mechanical hypersensitivity, the combination of sleep fragmentation and musculoskeletal sensitization had dramatic and long-lasting effects on subsequent sleep-wake behavior (Figures 9, 10). Before any manipulations, mice used in group 3 exhibited normal diurnal distributions of sleep-wake behavior during baseline recording periods, with increased time spent in NREM and REM sleep during the light period and increased time spent in wakefulness during the dark period (data not shown). To examine the impact of experimental manipulations, data obtained from mice among treatment groups were normalized to the pre-injection baseline measurements for the 12h light and dark periods and are expressed as the percent change from baseline ([post-manipulation value / baseline value] x 100). Because von Frey testing is

disruptive to spontaneous sleep and occurred early in the light period of post-sensitization days 1, 3, 7, 14, and 21, sleep-wake behavior was determined from recordings that were obtained on post-sensitization days 2, 8, 15, and 22 from undisturbed mice.

Following sleep fragmentation combined with musculoskeletal sensitization, sensitized mice had a significant increase in sleep-wake state transitions. Increased sleep-wake state transitions were apparent during the light and during the dark periods, and differed statistically from pre-injection baseline values and from control mice injected with normal saline (Figure 9A,B). These effects were robust, and persisted for the duration of the 22 day post-sensitization period evaluated in this study.

NREM sleep and wakefulness were altered during the post-manipulation period in mice that had been subjected to sleep fragmentation during musculoskeletal sensitization (acidified saline injections; Figure 10). The amount of time spent in REM sleep during the post-manipulation period did not differ among conditions, although there was a trend towards increased REM sleep during the light period on post-sensitization day 2 (Figure 10A). The combination of sleep fragmentation and musculoskeletal sensitization had differential effects on NREM sleep of mice during the post-manipulation period. NREM sleep of sensitized mice was reduced during the light period across all recording days evaluated, and increased during the dark period on post-manipulation days 15 and 22 (Figure 10B). By comparison, mice injected with normal saline and subjected to sleep fragmentation had a modest increase in NREM sleep that was

restricted to the dark period of post-manipulation day 2 (Figure 10B). Wakefulness was significantly increased in sensitized mice during the light period on all post-manipulation recording days (Figure 10C). Sensitized mice also had a significant decrease in wakefulness during the dark period on post-manipulation days 15 and 22. There were no significant changes in REM sleep or wakefulness of mice injected with normal saline (Figure 10A,C).

Because of inter-animal variations in properties of the recorded EEG, analyses of NREM delta power were performed on values normalized to the 24-hour average for each animal ([hourly value / 24 hour average] x 100)(23). These values were then expressed as the percent change from baseline ([post-sensitization 12h normalized value / baseline 12h normalized value] x 100). The combination of sleep fragmentation and musculoskeletal sensitization increased NREM delta power during the light period and decreased NREM delta power during the dark period. These effects were most apparent on post-manipulation days 8, 15, and 22 (Figure 10D). There were no changes in NREM delta power of mice injected with normal saline during the musculoskeletal sensitization period.

Sleep fragmentation combined with musculoskeletal sensitization did not significantly alter daily food consumption, water consumption, or body weight. Repeated-measures ANOVA did not reveal a significant group or time effect on these clinical parameters (data not shown).

DISCUSSION

Results of this study demonstrate that disrupting sleep of mice during the light periods of the inter-injection interval required to induce musculoskeletal sensitization exacerbates mechanical hypersensitivity and alters subsequent sleep-wake behavior. The combined effects of sleep fragmentation and musculoskeletal sensitization on sleep-wake behavior include increases in the number of sleep-wake state transitions, alterations in NREM sleep and wakefulness, and in delta power during NREM sleep. Our data suggest that exacerbated mechanical hypersensitivity and changes in sleep-wake behavior under the conditions of this study are the result of a synergistic effect of sleep fragmentation combined with musculoskeletal sensitization. Importantly, these data also demonstrate that in this pre-clinical model, sleep fragmentation exacerbates pain as manifest by prolonged induction of mechanical hypersensitivity.

Data from this study demonstrate that sleep fragmentation combined with musculoskeletal sensitization increases the number of sleep-wake state transitions of mice during the light and dark periods for at least three weeks following sensitization. We previously demonstrated that musculoskeletal sensitization by itself increases state transitions of sensitized mice during the light period, but not the dark period(116). Increased numbers of state changes reflect poor sleep quality, and have been reported in several pre-clinical pain studies, including those of neuropathic and arthritic pain(161, 165, 199). Collectively, results of our previously published study(116) and these new data

suggest that sleep fragmentation combined with musculoskeletal sensitization exacerbates effects on sleep quality relative to responses to either manipulation alone. These pre-clinical results contribute to a growing literature demonstrating that musculoskeletal sensitization fragments sleep. Clinically, fibromyalgia is a chronic pain condition of unknown etiology that is characterized by fragmented sleep and musculoskeletal pain(221, 222). Sodium oxybate, a medication that consolidates sleep, improves subjective pain ratings in fibromyalgia patients(241, 242). Consolidation of sleep may improve pain symptoms, especially in patients with ongoing musculoskeletal pain(175). Additional studies are necessary to determine if sleep consolidation in this pre-clinical model would ameliorate pain symptoms associated with musculoskeletal sensitization.

Musculoskeletal sensitization by itself, i.e., without concurrent sleep fragmentation, does not alter the amount of time mice spend in NREM sleep, REM sleep or wakefulness, or change delta power during NREM sleep(116). A novel finding of this study is that when sleep fragmentation is combined with musculoskeletal sensitization, each of these parameters is altered. Furthermore, these effects are prolonged, and persist for at least three weeks. NREM sleep of mice subjected to sleep fragmentation combined with musculoskeletal sensitization is increased during the dark period and reduced during the light period (this study). Reductions in NREM sleep are reported in other pre-clinical pain models, including nerve constriction injury(161, 166), osteoarthritis(199) and nerve ligation(200). In our present study, changes in NREM sleep are mirrored by increased wakefulness during the light period and reduced wakefulness

during the dark period. These changes in NREM sleep and wakefulness suggest insufficient sleep during the light period, which is compensated by a NREM sleep rebound during the dark period. However, delta power during NREM sleep is increased during the light period and reduced during the dark period. Although NREM sleep duration and delta power during NREM sleep may change in parallel, there is ample literature demonstrating dissociation between these two parameters under a variety of conditions [reviewed(24)]. Our data demonstrate that in this model of musculoskeletal sensitization not only are changes in NREM sleep duration and delta power during NREM sleep dissociated, but that the relationship between NREM sleep duration and delta power during NREM sleep is very complex. Ample literature demonstrates that NREM delta power generally increases with duration of prior wakefulness [reviewed(24)]. Therefore, increased NREM delta power during the dark period is one anticipated consequence of insufficient sleep during the light period. This is not the case for data obtained from mice in this study subjected to sleep fragmentation during the musculoskeletal sensitization period. The precise mechanisms underlying the reciprocal changes in NREM sleep and delta power during NREM sleep in this model remain to be elucidated.

REM sleep deprivation of humans or rodents enhances pain across sensory modalities including thermal, mechanical, chemical and electrical stimuli(176, 181, 183). Conversely, REM sleep is reduced in animals subjected to some pre-clinical pain models, such as gouty arthritis(163), diabetic neuropathy(243), or orofacial pain(218). Observations such as these suggest a

link between REM sleep and pain symptoms such that reduced REM sleep enhances pain, and/or enhanced pain reduces REM sleep. Data obtained in this study demonstrate that sleep fragmentation by itself, i.e., in the absence of musculoskeletal sensitization, does not induce mechanical hypersensitivity. Although our sleep fragmentation method does not dramatically alter NREM sleep, REM sleep is essentially abolished during periods when the disc is rotating(65). Our finding that sleep fragmentation by this method does not induce mechanical hypersensitivity may be important as studies of humans and rodents that use total sleep deprivation(177, 238), REM sleep deprivation(171, 180, 183), or sleep disruption(244, 245) report increases in pain symptoms using other outcome measures. Ongoing studies aim to understand the impact of sleep disruption by this method on multiple aspects of rodent physiology and behavior, including pain symptoms. This initial study using this method and protocol to disrupt sleep of mice suggests that five days of REM sleep loss during the light period may not have the same impact on pain symptoms in otherwise healthy rodents as reported in some studies that used other approaches to eliminate or disrupt sleep, or in other pain models.

Musculoskeletal sensitization does not damage peripheral tissue, and mechanical hypersensitivity in this model is mediated by changes in the central nervous system(110, 118). Sleep fragmentation combined with musculoskeletal sensitization enhances mechanical hypersensitivity to a greater extent than that elicited by musculoskeletal sensitization alone, i.e., there is an exacerbated response. Our data indicate that the combination of sleep fragmentation and

musculoskeletal sensitization induces mechanical hypersensitivity at least two days earlier than when musculoskeletal sensitization occurs in mice allowed undisturbed sleep. In addition, the exacerbated mechanical hypersensitivity is apparent 21 days after sensitization. One possible explanation for the exacerbated increase in mechanical hypersensitivity three weeks after sensitization is the change in sleep that occurs during this period. In human subjects, sleepiness increases subjective pain and lowers thresholds for evoked pain responses(192, 193). Three weeks after sensitization, sleep-wake behavior of mice during the light period is altered such that NREM sleep duration is reduced, there is more wakefulness, and the number of sleep-wake state transitions is increased. These changes in sleep of mice indicate sleep of poor quality that fundamentally differs from sleep during baseline conditions prior to musculoskeletal sensitization. Although the manipulations used in this study induce complex changes in NREM delta power and NREM sleep duration, these data suggest that in mice the quality of sleep contributes to pain perception. Within this context, musculoskeletal sensitization combined with sleep fragmentation may model aspects of the relationship between sleep and pain reported in human subjects(7, 17, 55).

Sleep deprivation, disruption, and fragmentation can all contribute to a systemic proinflammatory state(63, 246, 247). Circulating proinflammatory cytokines increase under conditions of disrupted sleep in rodents and human subjects(247-249). Sleep fragmentation may thus contribute to an inflammatory state that alters outcomes of musculoskeletal sensitization. Unpublished data

from our laboratory demonstrate that sleep fragmentation of mice during the light period by the method used in this study increases proinflammatory cytokines in plasma and discrete brain regions. Sleep fragmentation by this method also enhances the febrile response of mice to an intraperitoneal lipopolysaccharide (LPS) injection(65). Lipopolysaccharide, an endotoxin found in the membrane of gram-negative bacteria, induces a systemic inflammatory response. Musculoskeletal sensitization can be induced by replacing the first intramuscular injection of acidified saline with a systemic injection of LPS, suggesting inflammation is a critical component of musculoskeletal sensitization(250). An LPS injection into rats that precedes intramuscular injection of acidified saline by 5 days is sufficient to induce mechanical hypersensitivity that is similar in magnitude to that of animals subjected to 2 intramuscular injections of acidified saline(250). Ongoing studies in our laboratory focus on the role of inflammatory mediators in this model of sleep fragmentation combined with musculoskeletal sensitization.

Bi-directional relationships among multiple facets of sleep and pain exist such that pain disrupts sleep and insufficient sleep contributes to pain. The bi-directional relationships between sleep and pain may be important clinically. Sleep restriction to three and a half hours of sleep per night for a week in healthy volunteers produces pain sensitivity comparable to that of patients suffering from chronic pain conditions(172). After a period of recovery sleep, pain measures from these subjects return to normal(172). Healthy subjects who report sleeping more than 9h or less than 6h per night have greater next-day pain scores(174).

Increasing the sleep opportunity of healthy individuals by 2h a night for 4 nights significantly raises pain thresholds to a radiant heat stimulus(193). Sleepiness also impacts the efficacy of common treatments for pain. Patients given a sleep aid as part of post-surgical care have less subjective pain and require fewer analgesic doses to control pain than patients without a sleep aid(251, 252). Sleep deprived rats are resistant to the effects of opioid drugs that enhance tolerance to noxious stimuli, and do not receive the same analgesic benefits from these agents as well-rested rats(253, 254). The decreased efficacy of opioids in sleep-deprived rats may be clinically important given the widespread use of these agents to treat musculoskeletal pain(101, 255). Patients with chronic musculoskeletal pain often have poor sleep quality(102, 256). Pre-clinical data suggest that poor sleep quality, in turn, impacts the effectiveness of opioids in reducing pain. Conversely, acute opioid administration negatively impacts sleep quality in humans(257). Chronic administration of opioids has deleterious effects on sleep and contributes to the development of disordered breathing that can fragment sleep(101, 258). Collectively, these data raise the possibility that musculoskeletal pain fragments sleep, which could further enhance pain and make patients resistant to opioid treatment contributing to an even further deterioration in sleep quality. This complex interrelationship among sleep, pain and opioids, a drug class commonly prescribed for the treatment of pain, further underscores the need for research focused on insufficient sleep, chronic pain, and analgesic administration.

In conclusion, our data demonstrate that in this model, sleep fragmentation combined with musculoskeletal sensitization induces prolonged effects on mechanical hypersensitivity and sleep-wake behavior of mice. These effects result from synergistic interactions between sleep fragmentation and musculoskeletal sensitization, and do not result from sleep fragmentation or from musculoskeletal sensitization per se. Given the prevalence and increasing incidence of insufficient sleep in the United States, the relationship between chronic pain and sleep will continue to be a prominent public health issue. Additional pre-clinical, translational, and clinical investigations are needed so that the quality of life for the millions of individuals suffering these conditions may be improved.

FIGURES

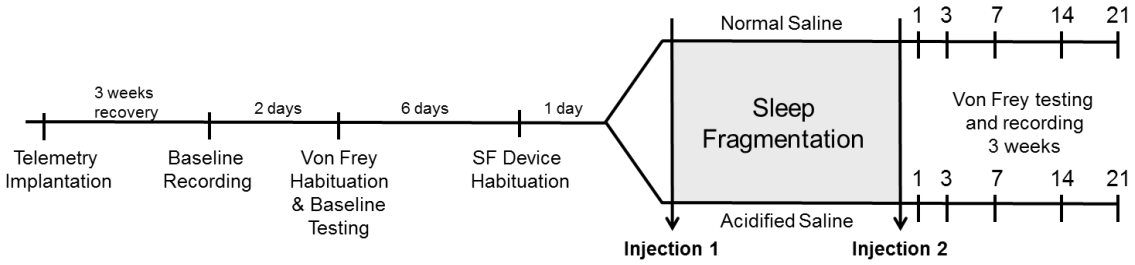

Figure 4. Experimental protocol to determine the impact of sleep fragmentation in combination with musculoskeletal sensitization on mechanical hypersensitivity and subsequent sleep-wake behavior of mice

Mice in group 3 (n=22) were used to determine the impact of sleep fragmentation combined with musculoskeletal sensitization on mechanical hypersensitivity. These mice were subjected to sleep fragmentation during the 5 day interval between the first and second intramuscular injections. A subset of mice (n=16) was used to determine the impact of sleep fragmentation combined with musculoskeletal sensitization on subsequent sleep-wake behavior. These n=16 mice were implanted with telemeters, and allowed 3 weeks of recovery. Baseline EEG and body temperature recordings were then obtained for two days. All mice (instrumented, uninstrumented) were habituated to the von Frey testing procedures, which was then followed by one day of habituation to the sleep disruption device. Mice then were randomized into an acidified or normal saline injection group (n=11 per injection; n=8 with telemeters per injection group). The first injection with acidified or normal saline was given at light onset, which was followed by sleep fragmentation for 5 consecutive light periods before the second injection was given. Testing with von Frey filaments took place on post-sensitization days 1, 3, 7, 14, and 21.

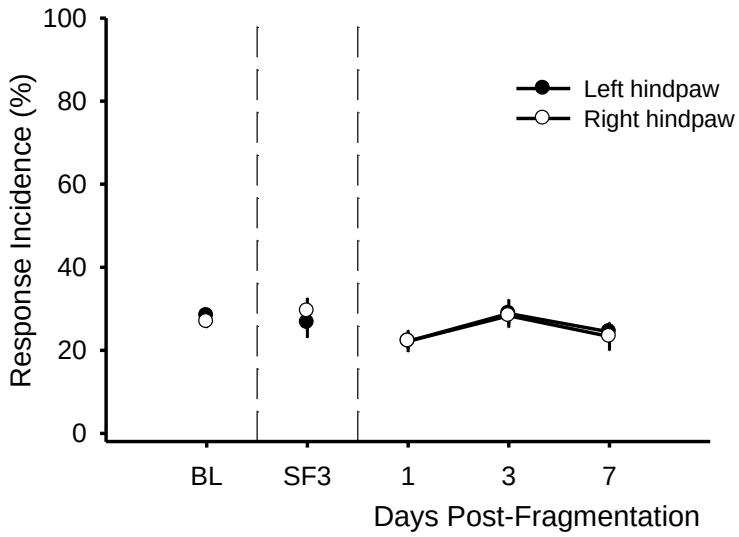

Figure 5. Sleep fragmentation by itself does not induce mechanical hypersensitivity

Mice in group 1 (n=12) that had sleep fragmented for 5 consecutive light periods (days) did not develop mechanical hypersensitivity. Response incidence to von Frey filament presentation did differ among baseline (BL), sleep fragmentation day 3 (SF3), or post-fragmentation days 1, 3, and 7 in either the left or right hindpaw. Responses to von Frey filaments are plotted as mean ± SEM percent of total response incidence ([total responses / total filament presentations] x 100) per paw.

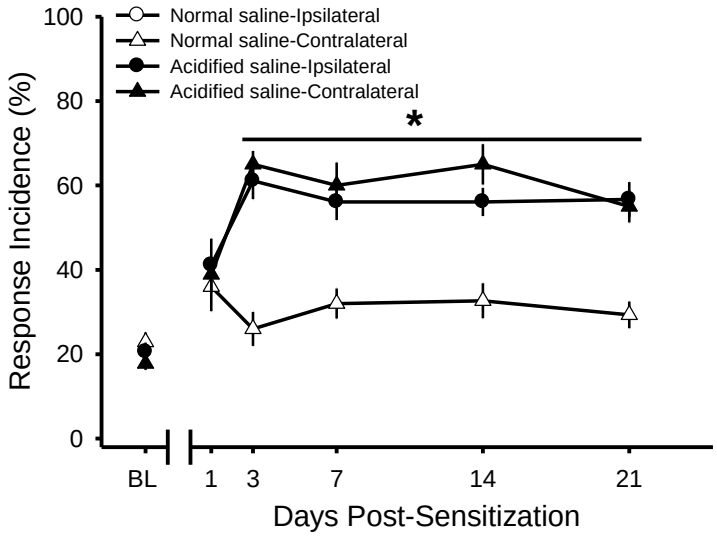

Figure 6. Musculoskeletal sensitization induces bilateral mechanical hypersensitivity

Mechanical hypersensitivity in mice was induced by two injections of acidified saline spaced 5 days apart. Mechanical hypersensitivity manifested as increased response incidence to von Frey filaments on post-sensitization days 3 through 21. Mechanical hypersensitivity developed to the same extent in the hindpaw contralateral to the leg that contained the intramuscular injection site. Responses to von Frey filaments are plotted as mean ± SEM percent of total response incidence ([total responses / total filament presentations] x 100) per paw from n=24 mice (n=12 acidified saline, n=12 normal saline). *, $p \leq 0.05$ vs. normal saline for both contralateral and ipsilateral hindpaws.

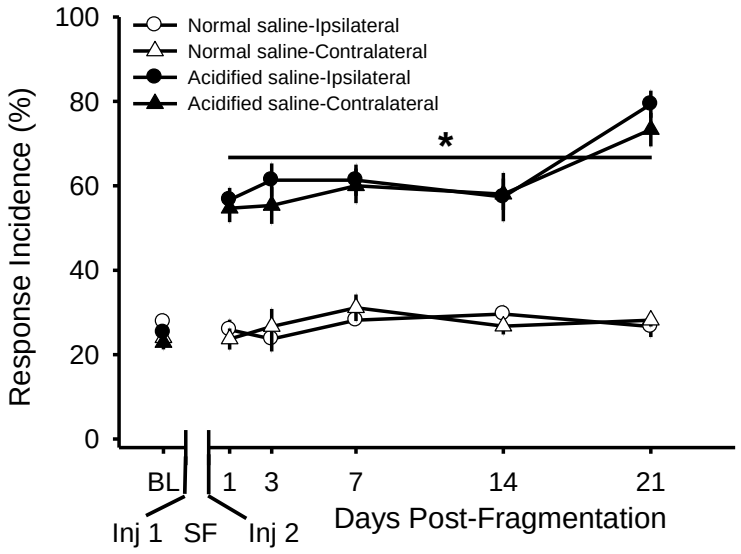

Figure 7. Sleep fragmentation combined with musculoskeletal sensitization induces bilateral mechanical hypersensitivity

Mice in which sleep was fragmented during the musculoskeletal sensitization period developed mechanical hypersensitivity that lasted for at least 21 days. . Mechanical hypersensitivity developed to the same extent in the hindpaw contralateral to the leg that contained the intramuscular injection site. Responses to von Frey filaments are plotted as mean ± SEM percent of total response incidence ([total responses / total filament presentations] x 100) per paw from n=22 mice (n=11 acidified saline, n=11 normal saline; n=8 mice in each injection group had implanted telemeters). BL = baseline; SF = sleep fragmentation; Inj = injection. *, $p \leq 0.05$ vs. normal saline.

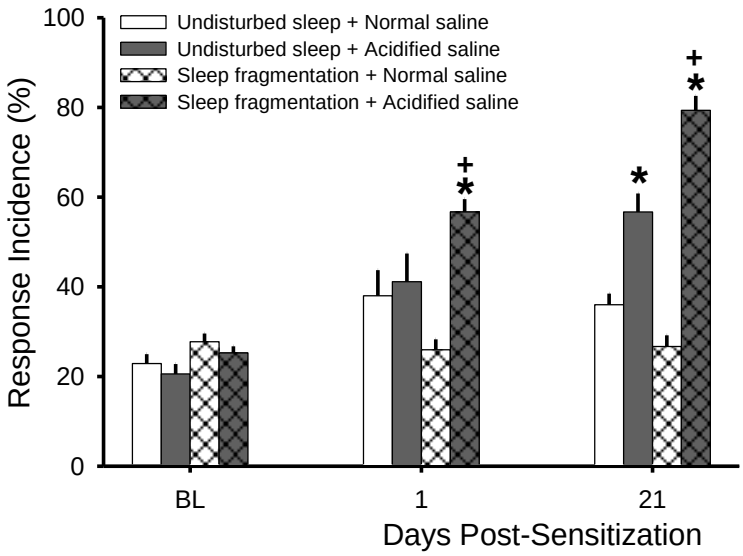

Figure 8. Sleep fragmentation combined with musculoskeletal sensitization exacerbates mechanical hypersensitivity

Comparisons were made between mice allowed undisturbed sleep during the musculoskeletal sensitization period (group 2) and mice in which sleep was fragmented during the sensitization period (group 3). Response incidence values did not differ among manipulation groups at baseline (BL). Mechanical hypersensitivity did not develop in mice injected with normal saline, irrespective of whether or not sleep was undisturbed or fragmented. Mice subjected to sleep fragmentation during the musculoskeletal sensitization period exhibited greater mechanical hypersensitivity on the day 1 and day 21 post-sensitization than did mice that were sensitized without sleep fragmentation. Responses to von Frey filaments are plotted as mean ± SEM percent of total response incidence ([total responses / total filament presentations] x 100) per paw for the leg ipsilateral to the injection site. +, $p < 0.05$ vs. undisturbed sleep + acidified saline. *, $p < 0.05$ vs. normal saline of the same sleep manipulation.

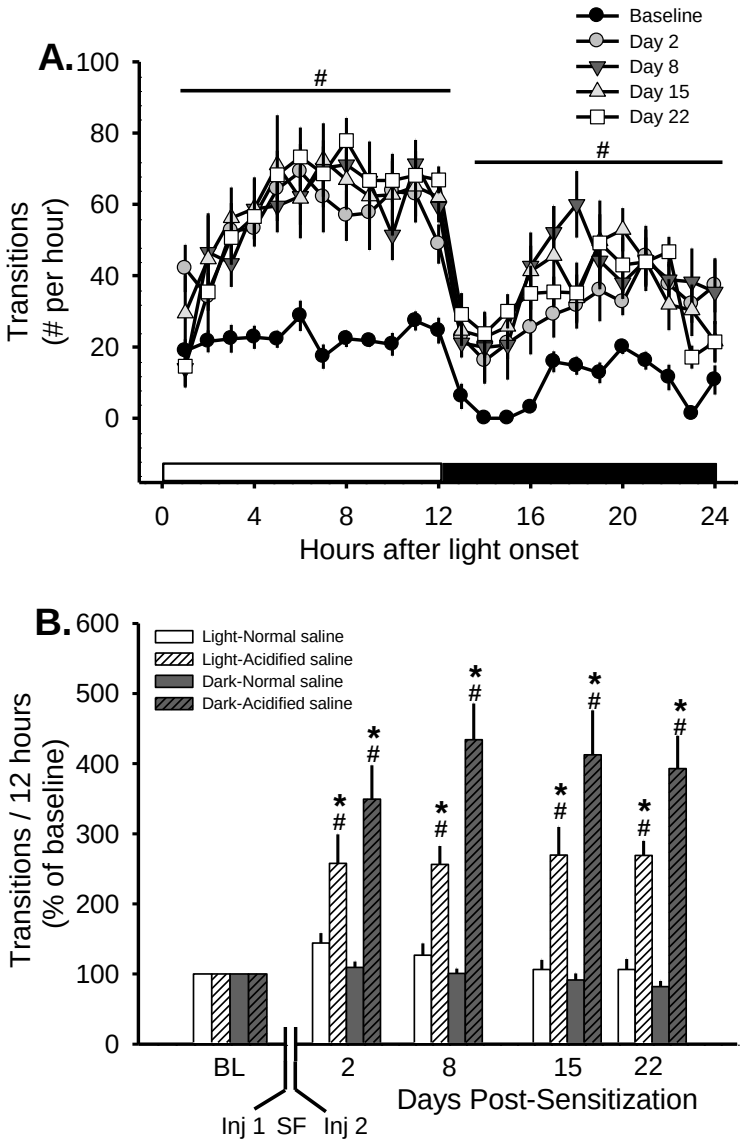
A.
Baseline
Day 2
Day 8
Day 15
Day 22
Transitions
(# per hour)
Hours after light onset
B.
Light-Normal saline
Light-Acidified saline
Dark-Normal saline
Dark-Acidified saline
Transitions / 12 hours
(% of baseline)
BL
2
8
15
22
Days Post-Sensitization
Inj 1
SF
Inj 2

Figure 9. The combination of sleep fragmentation and musculoskeletal sensitization disrupts subsequent sleep for prolonged periods

(A) The average number of sleep-wake state transitions per hour across the 24h light:dark period is plotted for pre-injection baseline and post-manipulation days 2, 8, 15, and 22. All mice were subjected to sleep fragmentation (SF) with or without musculoskeletal sensitization (acidified saline, normal saline injections). Sleep fragmentation combined with musculoskeletal sensitization significantly increased state transitions per hour during the light and during the dark periods. (B) The percent change from baseline (BL) for the 12h light and the 12h dark period is plotted for post-sensitization days 2, 8, 15, and 22. On all post-manipulation days, mice with musculoskeletal sensitization (acidified saline) had significantly more sleep-wake state transitions than mice without musculoskeletal sensitization (normal saline), an effect apparent during the light period and during the dark periods. Values are the mean ± SEM for n=7 mice per injection group. #, $p \leq 0.05$ vs. pre-injection baseline; *, $p \leq 0.05$ vs. normal saline.

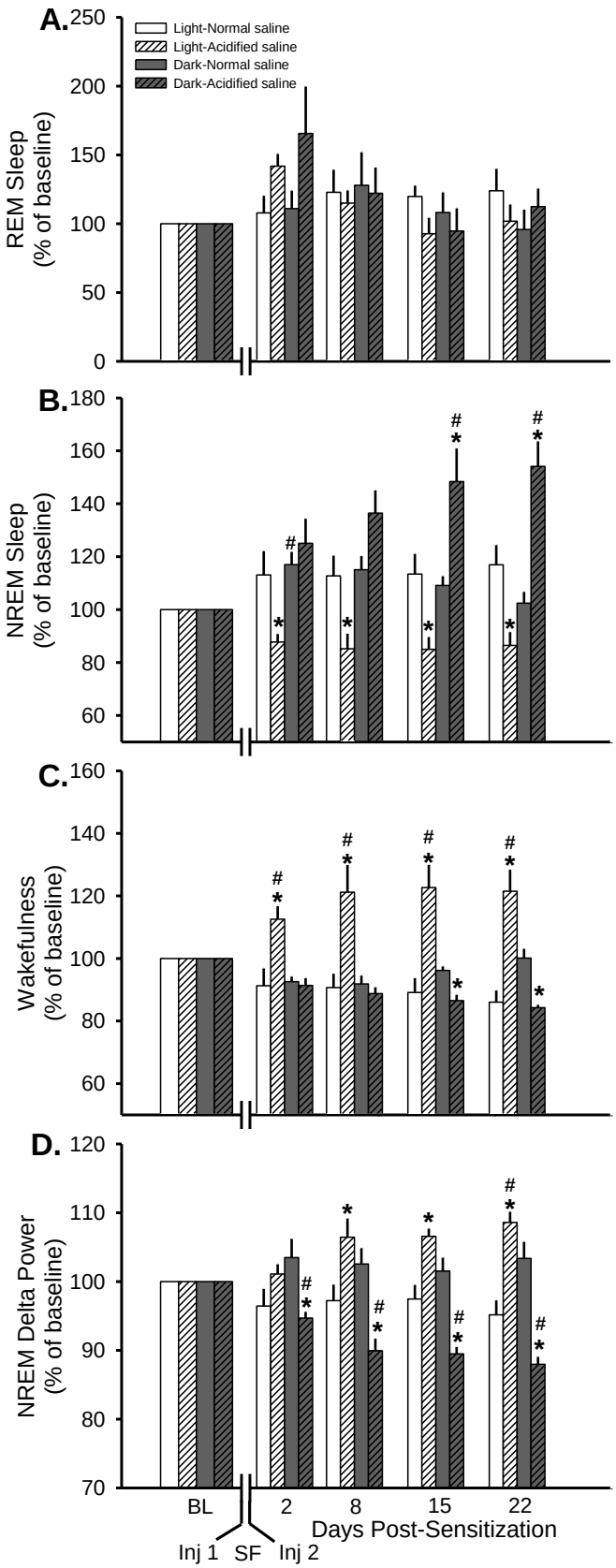
A.
Light-Normal saline
Light-Acidified saline
Dark-Normal saline
Dark-Acidified saline
REM Sleep (% of baseline)
250
200
150
100
50
0
B.
NREM Sleep (% of baseline)
180
160
140
120
100
80
60
C.
Wakefulness (% of baseline)
160
140
120
100
80
60
D.
NREM Delta Power (% of baseline)
120
110
100
90
80
70
BL
2
8
15
22
Days Post-Sensitization
Inj 1
SF
Inj 2

Figure 10. The combination of sleep fragmentation and musculoskeletal sensitization alters duration and quality of subsequent sleep

The impact of sleep fragmentation (SF) during the musculoskeletal sensitization period on rapid eye movements (REM) sleep, non-REM (NREM) sleep, wakefulness, and delta power during NREM sleep is presented as percent change from baseline (BL) values. Values are the mean ± SEM for n=7 mice per injection group. **(A)** Although there was a trend for increased REM sleep on the second post-sensitization day, this deviation did not achieve statistical significance (light period $p = 0.716$; dark period $p = 0.556$). REM sleep was not altered after sleep fragmentation combined with musculoskeletal sensitization. **(B)** NREM sleep of mice subjected to the combination of sleep fragmentation and musculoskeletal sensitization was decreased during the light period across all post-manipulation days, and increased during the dark period on post-manipulation days 15 and 22. **(C)** Wakefulness during the light period of mice in which sleep fragmentation was combined with musculoskeletal sensitization increased during the entire post-sensitization period. Sensitized mice had significantly less wakefulness during the dark period on post-manipulation days 15 and 22. **(D)** NREM delta power in mice subjected to sleep fragmentation and musculoskeletal sensitization significantly increased during light periods and decreased during dark periods. For all panels, #, $p \leq 0.05$ vs. pre-manipulation baseline; *, $p \leq 0.05$ vs. normal pH saline injections.

CHAPTER IV
INTRAMUSCULAR INFLAMMATORY CYTOKINES ARE NECESSARY FOR THE DEVELOPMENT OF MECHANICAL HYPERSENSITIVITY IN A MOUSE MODEL OF MUSCULOSKELETAL SENSITIZATION

ABSTRACT

Musculoskeletal pain is a widespread health problem in the United States, with back pain, neck pain, and facial pain constituting three of the most prevalent types of chronic pain. Cytokines contribute to pain during a variety of pathologies. Despite its prevalence in the United States, preclinical research investigating musculoskeletal pain is limited. Musculoskeletal sensitization is a preclinical model of muscle pain that produces mechanical hypersensitivity. In a rodent model of musculoskeletal sensitization, mechanical hypersensitivity develops at the hindpaws after injection of acidified saline (pH 4.0) into the gastrocnemius. In this study, we investigate the role of intramuscular cytokines in the development of mechanical hypersensitivity after musculoskeletal sensitization. Intramuscular concentrations of interleukin-1β (IL-1), IL-6 and tumor necrosis factor-α (TNF) were quantified following injection of normal (pH 7.2) or acidified saline into the gastrocnemius muscle. A cell-permeable NF-κB inhibitor was used to determine the impact on mechanical hypersensitivity of inhibiting nuclear translocation of NF-κB prior to musculoskeletal sensitization. The role of individual cytokines in mechanical hypersensitivity following musculoskeletal sensitization was

assessed using knockout mice lacking components of the IL-1, IL-6 or TNF systems. Collectively, our data demonstrate that acidified saline injection increases intramuscular IL-1 and IL-6, but not TNF; that intramuscular pre-treatment with an NF-κB inhibitor blocks mechanical hypersensitivity; and that genetic manipulation of the IL-1 and IL-6, but not TNF systems, prevents mechanical hypersensitivity following musculoskeletal sensitization. These data establish that actions of IL-1 and IL-6 in local muscle tissue play an acute regulatory role in the development of mechanical hypersensitivity following musculoskeletal sensitization.

INTRODUCTION

Chronic pain is a significant health problem in the United States and is associated with high personal and economic costs. Chronic pain conditions involving musculoskeletal pain are particularly burdensome because low back pain, neck, and facial pain constitute three of the most prevalent chronic pain conditions in the United States(4). Total annual costs associated with chronic pain are estimated upwards of $635 billion in the United States alone, including treatment costs and lost work productivity(5). Pain is one of the leading causes of absenteeism from work and presents physical, social and psychological barriers to working(259). Pain control for patients with musculoskeletal pain is poor and drug dependency can occur from opioid treatment(101). These demographic and epidemiologic data underscore the need for understanding mechanisms by which chronic musculoskeletal pain develops. Such knowledge may lead to new or more effective treatment interventions and thus improve

quality of life for patients and reduce overall costs associated with this public health burden.

Data demonstrate that inflammatory cytokines are mediators and modulators of many pain conditions(260-262). The inflammatory cytokines interleukin-1β (IL-1), IL-6, and tumor necrosis factor-α (TNF) are all involved in muscle pain(134, 263). Elevated inflammatory cytokines are detected in plasma and tissue biopsies of patients with chronic pathologies characterized by muscle pain(264-266). Targeting inflammatory cytokines for inhibition is effective in relieving pain of patients with rheumatoid arthritis(267) and ankylosing spondylitis, a form of chronic inflammatory arthritis(268). Inhibition of cytokines also relieves pain in pre-clinical models of neuropathic pain(137, 269), spinal nerve ligation pain(135), and experimental arthritis(270). Mice lacking components of the IL-1, IL-6, or TNF systems through genetic knockout do not develop the same intensity of pain as genetically intact mice in models of gouty arthritis, chronic inflammation, and nerve transection(271-273).

Reduced tissue pH is a characteristic of painful conditions induced by inflammation, muscle spasm, exhausting exercise, cancer, and ischemia, for example(274-277). Intramuscular injection of acidified saline reduces local tissue pH by increasing extracellular hydrogen ion (H^+) concentrations, and injection of acidified saline into the gastrocnemius muscle of rodents is used to study chronic muscle pain(114). In this rodent model, musculoskeletal sensitization by injection of acidified saline produces long-lasting bilateral secondary mechanical hypersensitivity at the hind paws(115, 116, 278). This rodent model of

musculoskeletal sensitization is clinically relevant, in part, because intramuscular injection of acidified saline into human volunteers produces muscle hyperalgesia and referred pain(279).

In this study we focus on inflammatory cytokines as mediators of musculoskeletal sensitization-induced mechanical hypersensitivity. Specifically, we hypothesize that IL-1, IL-6, and TNF are critical for the development of mechanical hypersensitivity following musculoskeletal sensitization. To test this hypothesis, we quantified cytokine concentrations in the mouse gastrocnemius muscle after intramuscular injection with normal or acidified saline; we targeted transcription of these three cytokines by inhibiting nuclear translocation of NF-κB; and we used genetically modified mice lacking components of the IL-1, IL-6 or TNF systems to determine relative contributions of these cytokines to mechanical hypersensitivity after musculoskeletal sensitization. We now report that IL-1 and IL-6, but not TNF, increase in muscle after intramuscular injection of acidified saline. Local inhibition of nuclear translocation of NF-κB in the gastrocnemius muscle prior to musculoskeletal sensitization blocks the development of mechanical hypersensitivity; and genetic manipulation of the IL-1 and IL-6, but not TNF, systems prevents mechanical hypersensitivity following musculoskeletal sensitization. Collectively, our new data demonstrate that intramuscular IL-1 and IL-6 are local mediators of the development of mechanical hypersensitivity following musculoskeletal sensitization.

METHODS

Animals

Adult male mice (8-12 weeks; 25 g) were used in this study. Mice were either purchased from the Jackson Laboratory (Bar Harbor, ME), or bred in-house as detailed later. All mice were maintained on a 12:12 h light:dark cycle at 27 °C with ad libitum access to food and water. Mice were group housed, and mice shipped from the Jackson Laboratory were allowed a minimum of one week to acclimate after arrival in our animal facility. All procedures using mice in these studies were approved in advance by the University of Washington Institutional Animal Care and Use Committee (IACUC), in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals.

Musculoskeletal Sensitization

Acidified saline injections were used to produce long-lasting bilateral secondary mechanical hypersensitivity at the hindpaws(114-116). Mice were briefly anesthetized using isoflurane and injected unilaterally into the gastrocnemius muscle with 20 uL of pyrogen-free saline using a 31g insulin syringe. All mice were randomized into normal (pH 7.2) or acidified (pH 4.0) saline injection groups. Saline was adjusted to acidified (pH 4.0) pH using 0.1M HCl or NaOH. The leg into which injections were made was randomized among mice to prevent any lateralization bias. All animals were immediately returned to their home cage after injections and observed by the investigator until fully ambulatory.

Mechanical Hypersensitivity Testing

The von Frey filament test is used to measure sensitivity to a non-noxious punctate pressure stimulus. All habituation and testing procedures took place at light onset and were completed during the first 2 hours of the light period. Mice were habituated to a galvanized steel mesh testing platform for a minimum of 60 minutes for 3 days prior to baseline testing. During testing, mice were given a minimum of 30 minutes to habituate to the testing platform or until quiet. Calibrated filaments (0.07, 0.45, & 1.45 g) were presented in ascending order to the glaborous skin of the hindpaw until the filament bowed slightly for 3 continuous seconds(116, 117). Testing continued until all 3 filaments were presented 5 times per paw with a minimum of 1-minute break between filaments. If mice became too active, testing was suspended until they were quiet. Positive responses were recorded when mice retracted the paw in response to the filament pressure. Mice were tested on 3 baseline days and 1, 3, 7, 14, and 21 days after the second sensitization injection.

Experiment 1: Quantification of intramuscular cytokine concentrations after musculoskeletal sensitization

A total of 62 mice were used for this experiment. The gastrocnemius muscles ipsilateral and contralateral to the injection site were collected from all mice. Four mice that were not injected were sacrificed at light onset to serve as un-injected controls. Fifty-eight mice (n=29 per injection group) were injected intramuscularly at light onset with either 20 uL normal (pH 7.2) or acidified (pH 4.0) saline. Twenty-four mice (n=4 per time point per injection group) were sacrificed 60, 90, or 120 minutes after the first intramuscular injection (injection

1). The remaining 34 mice were housed for 5 days without manipulation. Five days later, 24 mice received a second injection of normal or acidified saline (injection 2). Muscle tissues were collected from these animals 60, 90 or 120 minutes after injection (n=4 per time point per injection group). The remaining mice (n=10; 5 per injection group) were sacrificed at light onset without a second intramuscular injection and served as controls for the second intramuscular injection procedure. All muscle tissues were rapidly dissected from the hind limb, skin removed, snap-frozen in liquid nitrogen, and stored at −80 °C until processing.

Protein extractions: Frozen tissues were thawed and disrupted in Bioplex cell lysis buffer (BioRad catalog # 171-304011) containing protease and phosphatase inhibitors (BioRad catalog # 171-304012) and the protease inhibitor phenyl-methylsulfonyl fluoride (PMSF, 500 mM; Sigma–Aldrich). Tissue was grossly dissected using surgical scissors, disrupted using a 1000 uL pipette with the tip cut off, and homogenized with a sonic dismembrator (Fisher, #FB-501-10). The homogenate was then agitated for 30–40 min on ice and centrifuged at 4 °C and 6000 × g for 20 min. The supernatant was removed, aliquoted and stored at −80 °C until assay. The protein content of each sample was determined using the bicinchoninic acid (BCA) assay (Pierce catalog # 23225, Rockford, IL), with bovine serum albumin (BSA) as a standard, according to the manufacturer's protocol.

Determination of cytokine concentrations: Custom bead sets were created in-house for all assays. xMAP Antibody coupling kits (Product #40-50016,

Luminex Corportation, Austin, TX), capture antibodies from antibody specific duo sets for murine IL-1, IL-6, and TNF (DY401E; DY406E; DY410E respectively; R & D Systems) and MagPlex microspheres for coupling (MC10034-YY, MC10038-YY, MC10065-YY; Luminex) were purchased. Beads were coupled to antibodies at a concentration of 5 ug of capture antibody per million beads. Briefly, reagents were brought to room temperature and beads were resuspended, pipetted into a reaction tube, and washed. Activation buffer was added, and the beads were rewashed. Sulfo-NHS, EDC, and activation buffer was added to the reaction tube and allowed to incubate for 20 minutes at 15-30 rpm on an orbital shaker. The microspheres were then washed a second time, activation buffer added again, then the appropriate capture antibody was added to the reaction tube and incubated for 2 hours at 15-30 rpm. Lastly, the beads were washed and stored at 4 °C protected from light until use.

All protein samples were run in duplicate in multiplexed assays. A total of 100 ug of muscle protein, as determined by the BCA assay, was loaded per well, and the volume brought to 50 ul per well using PBS with 1% BSA. Duo set recombinant standards for murine IL-1, IL-6, and TNF were serially diluted to create a 7-point standard curve. The concentration ranges for the standard curves were 27–20,000 pg/ml for each of the three cytokines.

The assays were run using a protocol similar to that used with commercially-available Bio-Plex kits. General practices included warming all reagents to room temperature prior to use, minimizing exposure of the beads to light by wrapping tubes and/or plates with aluminum foil, and using appropriate

agitation on a microplate shaker (600 rpm). Samples were incubated with the beads overnight at 4 °C. After warming to room temperature, a magnetic separator (Part #CN-0269-01, Luminex) was used to separate beads from the supernatant. Beads were washed and then incubated with detection antibodies (R&D Systems) for 30 minutes. After washing, the beads were incubated with phycoerythrin (#S866, Invitrogen) for 30 minutes and then washed a final time prior to analysis. The plates were read with a Bio-Plex 200 System, and the data analyzed using BioPlex Manager 4.1 software with five-parameter logistic regression (5PL) curve fitting. The goodness-of-fit for each point on the standard curve was determined by the BioPlex Manager software as a back-calculation of standards.

Experiment 2: Effects on mechanical sensitivity of intramuscular inhibition of NF-κB prior to musculoskeletal sensitization

A commercially available, cell-permeable peptide (SN50; EMD Millipore) was used to determine the impact of inhibition of nuclear translocation of NF-kB during musculoskeletal sensitization on mechanical sensitivity. Forty mice were randomized into control peptide (SM50; EMD Millipore) or inhibitor peptide groups and further randomized into a normal or acidified pH saline injection group. This randomization process yielded 4 manipulation groups (n=10 per group): control peptide + normal pH saline, control peptide + acidified pH saline, inhibitor peptide + normal pH saline, and inhibitor peptide + acidified pH saline. Prior to manipulation all mice were habituated to the von Frey testing platform and baseline values for mechanical hypersensitivity were obtained as described

earlier. After habituation and baseline von Frey testing, mice were randomized and injected intramuscularly at light onset with either the NF-κB inhibitor peptide or control peptide at a dose of 200 ng / 10 ul in saline. One hour later, the same muscle was injected with either normal (pH 7.2) or acidified (pH 4.0) saline per the randomization protocol (injection 1). The exact same procedure was repeated 5 days later (injection 2), with each mouse receiving the same substances as given for injection 1. Mechanical hypersensitivity was assessed on post-sensitization days 1, 3, 7, 14, and 21 using von Frey testing as described.

Experiment 3: The impact of genetic knockout of IL-1 receptor 1 (IL1R1), IL-6, or TNF receptor 1 (TNFR1) on musculoskeletal sensitization-induced mechanical sensitivity

Mice for this experiment were either purchased or bred in-house. C57BL/6J and B6.129S6-Il6^{tm1Kopf} (IL-6 knockout [IL-6 KO]) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-6 KO mice were originally generated on a 129S6 background(280), but have been backcrossed onto a C57BL/6J background for more than 11 generations. We have previously used these IL-6 KO mice in studies of responses to immune challenge(204, 281, 282). Three strains generated in-house were used: Il1r1$^{-/-}$ (IL-1R1 KO), Tnfr1$^{-/-}$ (TNFR1 KO), and wild type control (Il1r1$^{+/+}$ Tnfr1$^{+/+}$; WT). Mice with Il1r1 targeted null allele and Tnfr1 targeted null allele were acquired from the Jackson Laboratory as a double knockout strain B6;129S-Tnfrsf1a^{tm1Imx} Il1r1^{tm1Imx}/J (stock #003244). To have mutant and wild type control strains on the same genetic

background, we generated WT mice by crossing homozygous null Il1r1$^{-/-}$ with homozygous null Tnfr1$^{-/-}$ animals and then intercrossing their progeny. All three strains are on mixed genetic background (C57BL/6, 129S1/SvImJ, and SJL with about 50% C57BL/6). All mice bred in-house were genotyped prior to use in experiments.

Twenty mice per genetic strain were used for this experiment (n=100 total). Experiments began when mice were between 8-12 weeks old. All mice were habituated to the von Frey testing platform, and baseline mechanical hypersensitivity values using von Frey filaments were obtained as described above. After baseline von Frey testing, mice were randomized into either normal (pH 7.2) or acidified (pH 4.0) saline injection groups (n=10 mice per injection group per genotype), and two intramuscular injections were administered 5 days apart, as in Experiments 1 and 2. Following the second injection, mechanical hypersensitivity was assessed on days 1, 3, 7, 14, and 21.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows. All data are presented as mean ± standard error of the mean (SEM). Cytokine protein concentrations obtained in Experiment 1 were analyzed using a one-way ANOVA with manipulation (acidified saline, normal saline) as the fixed effect and protein (IL-1, IL-6, TNF) as the random effect. The Bonferroni correction was used for multiple comparisons to determine changes in cytokine protein across time. However, cytokine values were only analyzed for those time-points at which values were above the lower limit of detection (~27 pg/mL) of the custom bead

sets used in this study (see Methods Section). A p value ≤ 0.05 was accepted as indicating statistical significance for all analyses.

To determine if there was an effect of intramuscular injections, NF-kB inhibition or genetic manipulation of selected cytokine systems on mechanical sensitivity, comparisons were made by evaluating total response incidence ([total number of responses per paw / 15 possible responses per paw] x100) for the paw ipsilateral and contralateral to intramuscular injection. Repeated-measures ANOVA with the between-subjects factor of treatment or genotype and a within-subjects factor of time (post-sensitization day of the experimental protocol) was used to statistically evaluate response incidence values from ipsilateral and contralateral paws. A p value ≤ 0.05 was accepted for all statistical tests as indicating significant departures between the groups across the testing period.

RESULTS

Experiment 1: Quantification of intramuscular cytokine concentrations after musculoskeletal sensitization

Cytokine protein concentrations were below the limit of detection in all tissue samples taken from mice injected with normal pH saline and in all samples from the muscle contralateral to the injection site, irrespective of time point and/or treatment group (data not shown). Because cytokine concentrations in all control samples (no injection or injection with normal pH saline) were below the limit of detection, an arbitrary value of 0.5 pg/ml was assigned to these samples to allow statistical comparisons to be made. All samples were run twice on plates containing mixed sample conditions. IL-1 was significantly elevated across time

points after the first injection [$F(3,16) = 35542.959$, $p < 0.01$] and after the second injection [$F(3,14) = 20915.095$, $p < 0.01$] (Fig. 11A). IL-6 was significantly elevated in samples taken after the first injection [$F(3,11) = 1721.481$, $p < 0.01$], but samples taken after the second injection were all below the lower limit of detection (Fig. 11B). IL-6 is released from muscle tissue during stimulation, especially exercise, however the release of IL-6 rapidly adapts to repeated stimuli(97, 283). TNF was below the limit of detection in all samples (data not shown).

Experiment 2: Effects on mechanical sensitivity of intramuscular inhibition of NF-κB prior to musculoskeletal sensitization

Pre-injection baseline values obtained by von Frey testing indicated that mechanical sensitivity did not differ among mice subsequently randomized into one of the four injection groups (Fig. 12; [$F(3,86) = 0.228$, $p = 0.877$]). Repeated measures ANOVA revealed a significant effect of injection group on response incidence [$F(1,3) = 97.028$, $p < 0.01$], and post-hoc analysis identified the average response incidence of the control peptide + acidified saline injection group (42.8 +/- 1.3 %) as differing significantly from the other 3 injection groups (control peptide + normal saline, 15.5 +/- 1.3 %; inhibitor peptide + normal saline, 16.7 +/- 1.1 %; inhibitor peptide + acidified saline, 20.7 +/- 1.1 %) across the 3-week post-injection period (Fig. 12).

Experiment 3: The impact of genetic knockout of IL-1R1, IL-6, or TNFR1 on musculoskeletal sensitization-induced mechanical sensitivity

Response incidence did not differ between ipsilateral and contralateral sides irrespective of genotype or injection group (data not shown). Therefore, for ease of visual presentation only data from the leg ipsilateral to the injection site are presented. Similarly, pre-injection baseline response incidence values did not differ significantly among genotypes or injection groups.

IL1R1 KO mice: Mice lacking IL1R1 did not develop mechanical hypersensitivity after musculoskeletal sensitization. Repeated measures ANOVA revealed a significant effect of injection (normal pH vs. acidified pH; [$F(1,34) = 27.679$, $p < 0.01$]) and genotype (IL1R1 KO vs. WT; [$F(1,34) = 31.906$, $p < 0.01$] on response incidence across the 21 day post-sensitization testing period (Fig. 13A). WT mice in which musculoskeletal sensitization had been induced by injections of acidified saline developed mechanical hypersensitivity manifest by increased response incidence to von Frey filaments. There was a significant injection x genotype interaction on response incidence, indicating that IL-1R1 KO mice injected with acidified saline did not develop mechanical hypersensitivity (Fig. 13A; [$F(1,34) = 20.663$, $p < 0.01$]).

IL-6 KO mice: IL-6 KO mice did not develop mechanical hypersensitivity after musculoskeletal sensitization (Fig. 13B). Repeated measures ANOVA revealed a significant effect of injection on response incidence (normal pH vs. acidified pH; [$F(1,46) = 26.304$, $p < 0.01$]) across the 21 day post-sensitization period. There was a significant effect of genotype on response incidence (C57BL/6J vs. IL-6 KO; [$F(1,46) = 66.950$, $p < 0.01$]), and a genotype x injection

interaction demonstrating that IL-6 KO mice did not develop mechanical hypersensitivity following acidified saline injection [$F(1,46) = 11.113$, $p < 0.05$].

TNFR1 KO mice: Mice lacking TNFR1 developed mechanical hypersensitivity after musculoskeletal sensitization to the same extent as did WT mice (Fig. 13C). Repeated measures ANOVA revealed a significant effect of injection on response incidence across the 21 day post-sensitization period (normal pH vs. acidified pH; [$F(1,34) = 87.459$, $p < 0.01$]). There was no effect of genotype on response incidence (WT vs. TNFR1 KO; [$F(1,34) = 0.278$, $p = 0.601$]).

DISCUSSION

Results of these experiments provide data demonstrating that IL-1 and IL-6 play a critical role in the development of mechanical hypersensitivity after musculoskeletal sensitization. Injection with acidified saline causes an intramuscular increase of IL-1 and IL-6, and intramuscular inhibition of nuclear translocation NF-κB prevents mechanical hypersensitivity after musculoskeletal sensitization. Furthermore, genetic modification such that components of the IL-1 or IL-6 systems are lacking completely eliminates the development of mechanical hypersensitivity in this mouse model of musculoskeletal sensitization.

NF-κB is a transcription factor that regulates the expression of hundreds of genes(284) and is expressed ubiquitously in cells, including skeletal muscle(285). NF-κB, in part, regulates gene transcription of the inflammatory cytokines IL-1, IL-6 and TNF(284), making it a target for inhibition of transcription for all three cytokines. Our new data demonstrate that inhibiting nuclear

translocation of NF-κB locally in the muscle prevents the mechanical hypersensitivity that follows acidified saline injections. Inhibiting nuclear translocation of NF-κB also blocks pain in other pre-clinical models including corneal burn(286), trigeminal neuropathy(287), HIV induced inflammation(288), and post-ischemia pain(289, 290). Painful skeletal muscle disorders including Duchenne's muscular dystrophy are associated with increased NF-κB activity in humans(291). Duchenne's is also modeled pre-clinically using a genetic mouse model that has increased NF-κB activity and produces muscle pain(292). Our data contribute to the literature demonstrating a role of NF-κB in the regulation of chronic pain, and extend existing data by indicating that in this model local actions of NF-κB are critical to the development of musculoskeletal sensitization.

IL-1 and IL-6 increase in muscle tissue after injection of acidified saline, and knockout mice lacking IL-1R1 or IL-6 do not develop mechanical hypersensitivity after musculoskeletal sensitization. IL-6 is synthesized and released directly from skeletal muscle and is considered to be a myokine, or cytokine released from muscle that can contribute to pain(97). IL-1 increases intramuscular expression of acid sensing ion channels (see later). Extracellular acidosis increases the production of IL-1 in monocytes through the stimulation of pro-IL-1 mRNA synthesis, the precursor to IL-1(293). Our data clearly demonstrate a role for IL-1 and IL-6 as mediators of mechanical hypersensitivity in this model. It is possible based on *in vitro* studies demonstrating the selective release of IL-1 from monocytes and IL-6 from myocytes that the IL-1 and IL-6 detected during musculoskeletal sensitization is produced from different cell

types. In, contrast, TNF along with IL-1 were two of the first cytokines identified as enhancing nociception(294, 295) and there is ample literature supporting the role of TNF in other pain models(128, 296). However, our data also demonstrate that TNF does not play a role in this model: injection of acidified saline does not increase TNF in muscle tissue, and mice lacking TNFR1 develop mechanical hypersensitivity. Although mechanisms responsible for local cytokine actions within muscle tissue in this model remain to be fully elucidated, *in vitro* studies demonstrate that an acidic environment inhibits the release of TNF from macrophages in response to lipopolysaccharide stimulation(297). Intramuscular pH in the musculoskeletal sensitization model reaches roughly pH 6.5 post-injection with acidified saline(114). Thus, the acidic microenvironment in the gastrocnemius muscle after acidified saline injection may inhibit TNF release. Collectively, observations that IL-1 and IL-6, but not TNF, contribute to the development of mechanical hypersensitivity following musculoskeletal sensitization represent novel findings of this study.

Tissue acidosis, or an excess of hydrogen ions (H^+), can be caused by many factors, including but not limited to insufficient cardiac function and sickle cell crises, tissue inflammation, and tumor microenvironments(298). Each of these conditions produce acidosis that can cause pain(298). Two primary pathways activate inflammation in muscle tissue; one characteristic of infection(299, 300) and the other from muscle fatigue(283, 301). Infection in muscle tissue releases IL-1, IL-6, and TNF and activates the classical inflammatory pathways mediated by NF-κB(300, 302). Fatiguing exercise

triggers predominantly IL-6 release(97, 303). In human subjects, infusion of low pH buffer evokes symptoms characteristic of fatiguing muscle pain, including mechanical hyperalgesia and referred pain(276, 279, 304). Although previous studies investigated the role of inflammatory cytokines in maintaining mechanical hypersensitivity after musculoskeletal sensitization in spinal cord, they did not quantify cytokine secretion in spinal cord or muscle tissue(305). The effect of acidified saline injection into muscle has not been thoroughly investigated, but we detect IL-1 and IL-6, not TNF, in muscle tissue after injection. These data suggest that injection of acidified saline triggers inflammatory pathways independent of TNF and may model fatiguing exercise more so than classical inflammatory pathways associated with infection. Although this is one possible explanation for cytokine release in muscle tissue, it warrants further study to elucidate the specific mechanism of action.

Detection of H^+ occurs through two receptors types; transient receptor potential cation channel subfamily V member 1(306) and the acid sensing ion channels (ASIC). There are numerous ASIC channels, but ASIC3 is of particular interest because it is expressed exclusively on sensory neurons(307, 308). Inflammation increases the expression of ASIC3 on sensory neurons and therapeutic doses of non-steroidal anti-inflammatory drugs that reduce pain also prevent transcriptional increases of ASIC3(309). Previous data suggest that ASIC3 plays a role in the development, but not maintenance, of musculoskeletal sensitization(118, 310, 311). Pretreatment with a pharmacological antagonist for ASIC3, or genetic knockout of ASIC3, prevents mechanical hypersensitivity after

musculoskeletal sensitization. However, targeting ASIC3 after hypersensitivity has developed is ineffective in ameliorating mechanical hypersensitivity(118, 310, 311). IL-1 increases the expression of ASIC3 mRNA in dorsal root ganglion cell cultures(312). Although cytokines increase ASIC3 expression, a reciprocal role for ASIC3 in regulating cytokine expression is less clear. ASIC3 expression in bone marrow derived macrophages *in vitro* is required for the maturation of immune cells that can secrete cytokines(313). These data suggest the possibility that ASIC3 and inflammatory cytokines may have modulatory interactions, which in turn contributes to mechanical hypersensitivity. Although the specific mechanisms of interactions between ASIC3 and inflammatory cytokines are not well understood, ASIC3 could modulate the release of cytokines. This hypothesis remains to be tested.

Collectively, our new data demonstrate that inflammatory cytokines and the transcription factor NF-κB play a critical role in the development of mechanical hypersensitivity after musculoskeletal sensitization. Intramuscular injection with acidified saline causes a rapid rise in intramuscular IL-1 and IL-6 concentrations. Pre-treatment with an intracellular NF-κB inhibitor, or the genetic loss of IL-1R1 or IL-6 prevents the development of mechanical hypersensitivity. However, injection of acidified saline does not increase intramuscular TNF, and genetic loss of TNFR1 does not inhibit the effects of musculoskeletal sensitization. These data drive future research focused on inflammatory cytokines as mediators of musculoskeletal sensitization. Such research may

contribute to new treatments and help to reduce the high costs associated with musculoskeletal pain in the United States.

FIGURES

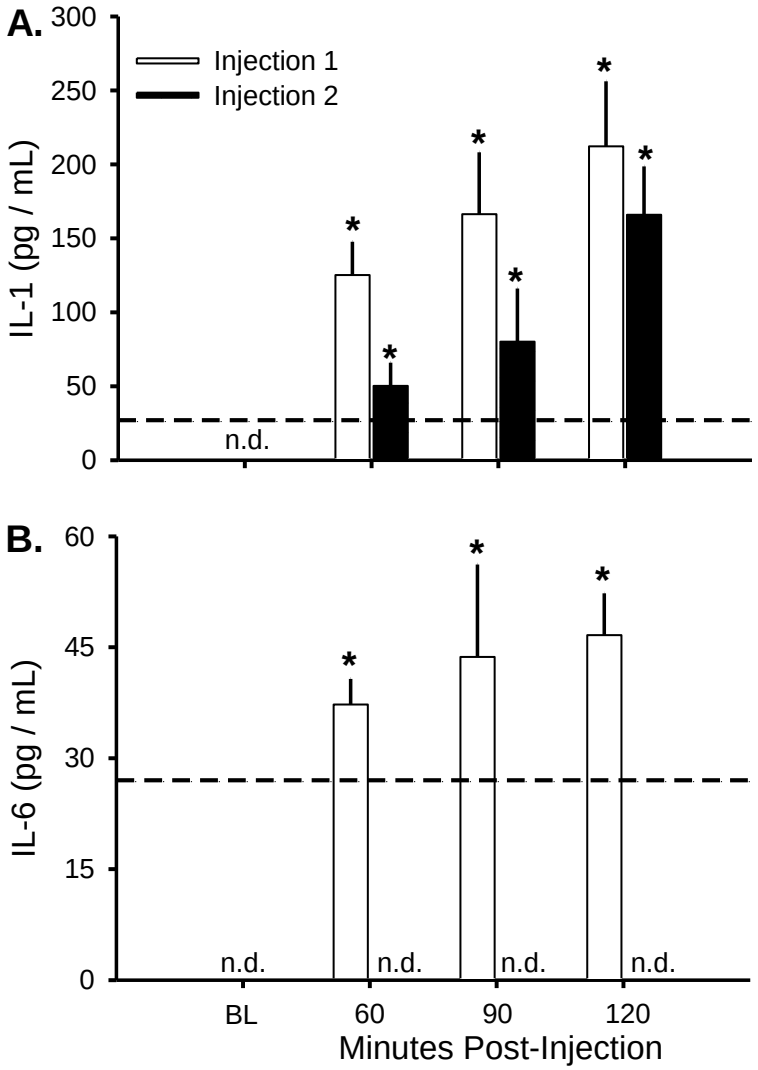
A.
300
250
200
150
100
50
0
Injection 1
Injection 2
IL-1 (pg / mL)
n.d.
*
B.
60
45
30
15
0
IL-6 (pg / mL)
n.d.
BL
60
90
120
Minutes Post-Injection

Figure 11. Musculoskeletal sensitization injections increase intramuscular IL-1 and IL-6

Data are plotted as the average cytokine concentration in pg/mL and presented only from the ipsilateral leg of mice injected with acidified saline. For statistical comparisons, the pre-injection time-point was set to an arbitrary low value of 0.5 pg/mL. (A) IL-1 is significantly increased in mice injected with acidified saline at all time-points after injection 1 and 2. (B) IL-6 is significantly increased across all three time-points after injection 1 but did not cross the threshold for detection after injection 2. n.d., cytokine concentration was not detectable and did not cross the lower threshold for detection. *, $p < 0.05$ vs. no injection on the same day.

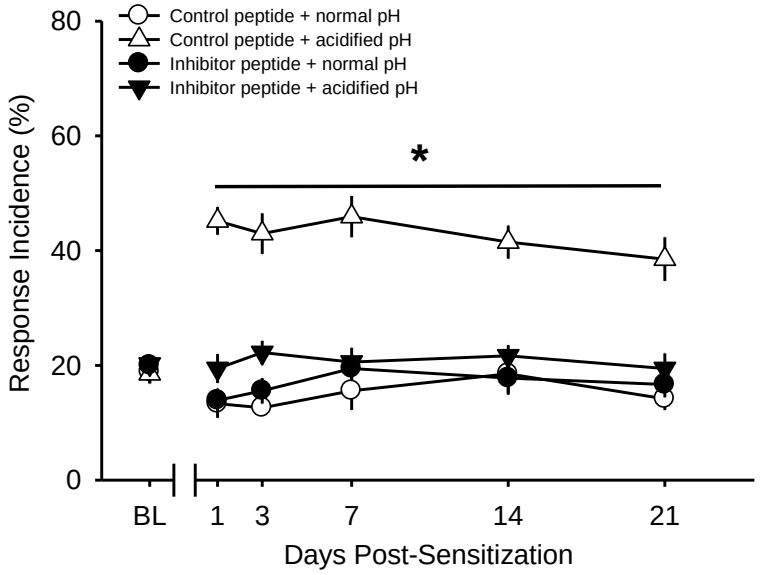

Figure 12. Intramuscular inhibition of NF-κB blocks mechanical hypersensitivity following musculoskeletal sensitization

The ipsilateral and contralateral leg for all injection groups did not significantly differ and data are presented only from the leg ipsilateral to injection (n=10 per treatment group). At pre-injection baseline the mice subsequently randomized into injection groups did not significantly differ. Injection with the control peptide + acidified saline did result in a significant increase in mechanical hypersensitivity. Inhibitor peptide + acidified saline injection did not develop mechanical hypersensitivity that differed from either normal saline injection group (Inhibitor or control peptide). Responsiveness to von Frey filaments are plotted as mean ± SEM of total response incidence percent ([total responses / total filament presentations] x 100) per paw. *, $p > 0.05$ of Control peptide + acidified pH.

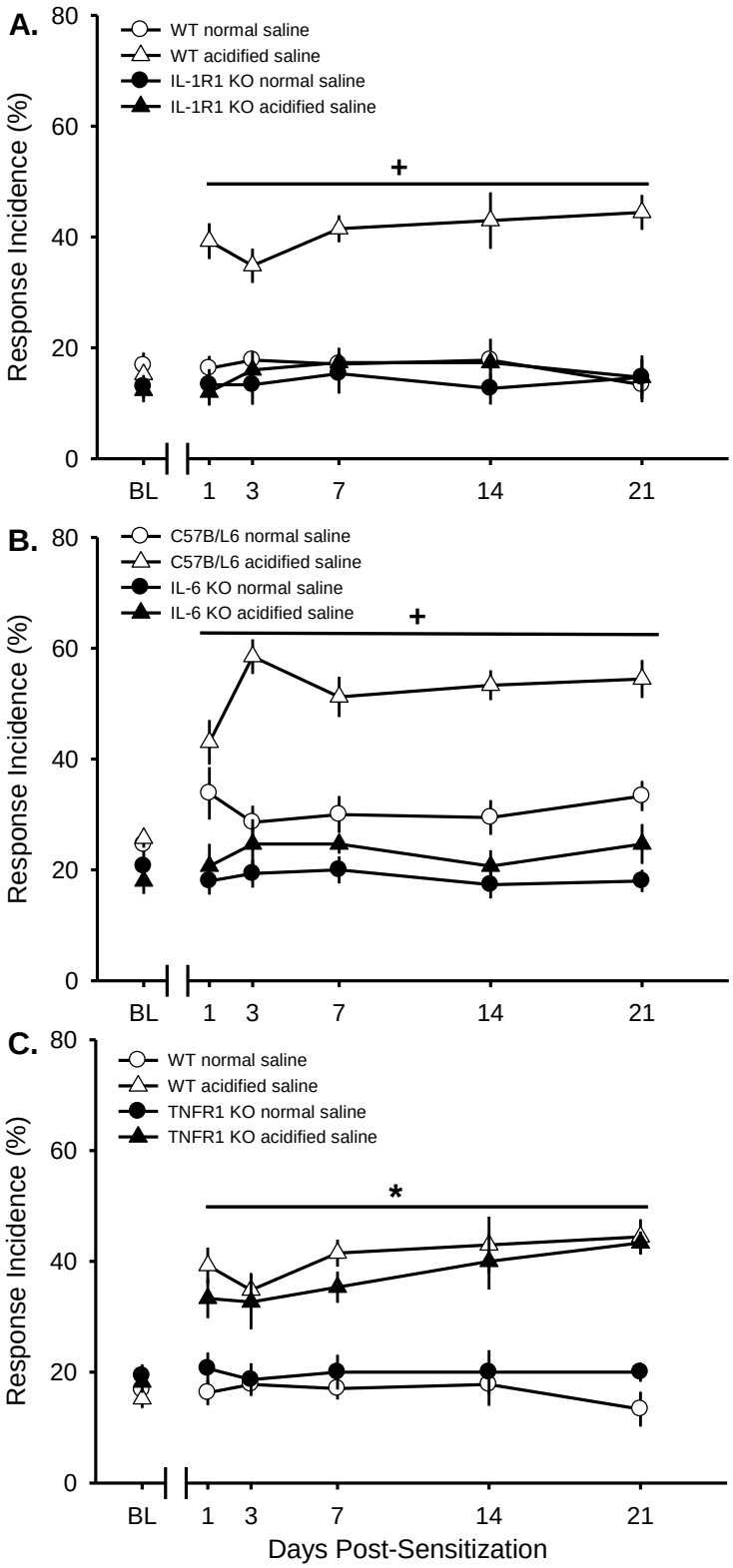
A.
WT normal saline
WT acidified saline
IL-1R1 KO normal saline
IL-1R1 KO acidified saline
+
Response Incidence (%)
0
20
40
60
80
BL
1
3
7
14
21
B.
C57B/L6 normal saline
C57B/L6 acidified saline
IL-6 KO normal saline
IL-6 KO acidified saline
+
Response Incidence (%)
C.
WT normal saline
WT acidified saline
TNFR1 KO normal saline
TNFR1 KO acidified saline
*
Response Incidence (%)
Days Post-Sensitization

Figure 13. IL-1R1 and IL-6 knockout mice do not develop mechanical hypersensitivity following musculoskeletal sensitization

Data are presented only from the leg ipsilateral to intramuscular injection (n=10 per group). For each panel knockout animals are plotted with the appropriate genetic control indicated in the figure legend. (A) IL-1R1 and (B) IL-6 knockout mice fail to develop mechanical hypersensitivity after injection with acidified pH saline. For both knockouts, the appropriate genetic controls develop mechanical hypersensitivity after injection with acidified saline. (C) TNFR1 knockout mice develop mechanical hypersensitivity after injection with acidified that is not significantly different from WT mice injected with acidified saline. Responsiveness to von Frey filaments are plotted as mean ± SEM of total response incidence percent ([total responses / total filament presentations] x 100) per paw. +, $p < 0.05$ interaction effect between injection and genotype. *, $p < 0.05$ effect of injection.

CHAPTER V
DISCUSSION

SUMMARY OF FINDINGS

The data of this dissertation provide evidence for the interactions among musculoskeletal sensitization, disrupted sleep, and inflammatory cytokines. Briefly, the data presented in the previous chapters are reviewed.

The quantification of sleep state in Chapter II demonstrates that for 3 weeks post-sensitization musculoskeletal sensitization increases the number of sleep state transitions during the light period without altering sleep duration. In Chapter III data demonstrate that fragmenting the sleep of mice for 5 days between the first and second intramuscular injections alters behavioral outcomes after musculoskeletal sensitization. Sleep fragmentation combined with musculoskeletal sensitization significantly increases state transitions of subsequent sleep during the light and dark periods for 3 weeks. The increase in state transitions is accompanied by changes in the duration of NREM sleep, duration of wakefulness, and delta power during NREM sleep. Sensitized mice that underwent SF are also hypersensitive to mechanical stimuli on day 1 and day 21 compared to sensitized mice with undisturbed sleep. Chapter IV demonstrates a role for intramuscular inflammatory cytokines in musculoskeletal sensitization. The inflammatory cytokines IL-1 and IL-6 significantly increase in muscle tissue injected with acidified, but not normal saline. Pharmacological

manipulation to block nuclear translocation of NF-κB prevents mechanical hypersensitivity following musculoskeletal sensitization. Lastly, genetic knockout of either IL-1R1 or IL-6, but not TNFR1, inhibits the development of mechanical hypersensitivity following musculoskeletal sensitization. Collectively, the data of this dissertation demonstrate that musculoskeletal sensitization increases the number of state transitions in the 3-weeks post-sensitization, intramuscular inflammatory cytokines play a mechanistic role in the development of mechanical hypersensitivity following musculoskeletal sensitization, and SF combined with musculoskeletal sensitization exacerbates the behavioral outcomes of sensitized mice including mechanical hypersensitivity and subsequent sleep.

LIMITATIONS AND FUTURE DIRECTIONS

The studies of this dissertation provide compelling data for the interactions of musculoskeletal sensitization and sleep; however, they are not without limitations. The objectives of this dissertation were to determine whether musculoskeletal sensitization or SF combined with musculoskeletal sensitization has an impact on subsequent sleep. Presumably, if alterations in sleep/wake behavior occur after musculoskeletal sensitization they would occur during the first 3 weeks post-sensitization. Previous studies using musculoskeletal sensitization demonstrate it takes between 6 and 12 weeks for mechanical hypersensitivity to terminate(114). The studies in this dissertation investigating sleep and mechanical hypersensitivity do not extend to include the resolution of mechanical hypersensitivity. Therefore conclusions cannot be drawn about the whether sleep disruption occurs in parallel or independently of mechanical

hypersensitivity. Future studies should investigate the full time course of effects for mechanical sensitization and sleep fragmentation.

Chapter IV also has technical considerations surrounding the use of genetically manipulated mice. IL-1R1 and TNFR1 knockout mice are receptor knockouts, while IL-6 is a ligand knockout. This difference in genetic manipulation is considered in the experimental design and additional pharmacological and protein assay data were collected. The results from the cytokine quantification assays and the genetic manipulations are consistent, and demonstrate that the IL-1 and IL-6, but not TNF systems playing a critical role in mechanical hypersensitivity. Another approach to investigate the role of cytokines in mechanical hypersensitivity would be the use of intramuscular pharmacological antagonists specific to IL-1, IL-6 and TNF. This would confine the area of manipulation to the injection leg rather than the whole animal approach using genetically manipulated mice. It would also enhance the cytokine specificity intramuscularly that the NF-κB inhibitor study did not provide. The technical considerations present opportunities for further research elucidating the specific mechanisms contributing to musculoskeletal sensitization and inflammatory cytokines.

One topic not addressed in the data of this dissertation is potential sex differences in response to musculoskeletal sensitization, as only male mice were used. Musculoskeletal pain in the human population is reported at roughly equal incidence rates in males and females(314), although females may be more sensitive to pressure pain associated with musculoskeletal discomfort(315). In

preclinical studies of chronic pain and sleep, differential responses are detected between male and female rodents. For example, preclinical studies of osteoarthritis in male and female rats detect that males have more disturbed sleep than females following osteoarthritis(198, 199). However, sex differences in pain and sleep varies widely depending on the pain model of investigation. An experimental model of temperomandibular joint inflammation detected pain and sleep disturbances in male rats, but no changes in female rats(316). Musculoskeletal sensitization has been induced in female CF-1 mice, however male mice were not investigated in the study and no sex differences in mechanical hypersensitivity can be inferred from the data(115). The responsiveness of males and females to painful mechanical stimuli may be the result of sexual dimorphism in the threshold of mechanical nociceptors, which was recently demonstrated *in vivo* and *in vitro* in rats(317). In female rats, the dorsal root ganglion cells that innervate the gastrocnemius muscle tissue have a significantly hyperpolarized resting potential compared with male rats(317). This increase in the resting potential of nociceptors innervating muscle tissue corresponds with a significantly higher threshold for the firing of action potentials in females compared with males(317). The sexual dimorphism of the response threshold of nociceptors innervating the gastrocnemius muscle tissue raises the possibility that musculoskeletal sensitization may have different effects in male and female rodents. The data of this dissertation provide data that the musculoskeletal sensitization model in male mice causes interactions between

sleep and mechanical hypersensitivity, however this relationship still needs to be investigated with female mice.

SLEEP AND PAIN INTEGRATION

The data presented in the previous chapters demonstrate a role for intramuscular inflammatory cytokines in the development of mechanical hypersensitivity following musculoskeletal sensitization. This is a novel finding, as mechanisms of musculoskeletal sensitization have not explored intramuscular actions but have focused on mechanisms in the spinal cord and brainstem(119, 278, 305). However, there are still questions surrounding the mechanisms maintaining the effects of musculoskeletal sensitization, particularly the interactions with sleep/wake behavior. The biological mechanisms independently underlying sleep and chronic pain are complex(111, 318). There are specific brain regions of intersection critical for the regulation of sleep and pain. The brainstem is the site of several arousal-promoting nuclei that send projections to higher cortical areas and contribute to wakefulness(319, 320). The brainstem also modulates pain by sending descending projections to the spinal cord and ascending projections to the amygdala and somatosensory cortices(113, 211, 321, 322). The rostral ventromedial medulla (RVM) regulates the peripheral sensory processing of painful stimuli(211). Injections of opioids, anesthetics, and cholinergic antagonists into the RVM can block a variety of painful stimuli, including mechanical hypersensitivity(110, 119, 214, 216, 217, 323). These data demonstrate the importance of the RVM in pain regulation during painful conditions including musculoskeletal pain.

The RVM regulates pain and sends projections to other nuclei associated with sleep. Histological studies of direct projections from the RVM identify outputs to a number of nuclei including the periaqueductal gray (PAG)(107, 108). The PAG is a nucleus of the midbrain that is adjacent to the cerebral aqueduct. The proximity of the PAG to the cerebrospinal fluid suggests a potential role in integrating between the central and peripheral nervous system. The PAG is one of the last nuclei in the descending pain pathway in the central nervous system(107, 108, 324). It also serves a function in sleep regulation and lesions of the PAG cause loss of consciousness(212, 325). In a rodent model of sciatic nerve injury, severity of pain and sleep disturbance is correlated with cell death in the PAG(166, 326). Although the PAG is one of numerous nuclei associated with pain, it is one of the few contributing to both sleep and pain. These data suggest that the PAG may warrant further investigation for its roles in sleep and pain during musculoskeletal sensitization.

ACIDOSIS MECHANISMS

The sensing of tissue pH both centrally and peripherally is critical to health. Changes in pH are associated with pathological conditions including ischemia, myopathies, and exhausting exercise(274). Two receptors detect hydrogen ions (H^+); TRPV1 and the ASIC family of receptors(327). TRPV1 is well characterized for its role in sensing capsaicin, the active ingredient in chili peppers, and TRPV1 has functions in thermoregulation and heat sensation(306). Of the ASIC family of receptors, ASIC3 is of interest as its expression is limited to sensory neurons in the peripheral nervous system(328). ASIC3 serves a critical role in the

development, but not the maintenance of mechanical hypersensitivity following musculoskeletal sensitization(118, 310, 311, 329). Pharmacological targeting of ASIC3 is not a viable therapeutic treatment for mechanical hypersensitivity because ASIC3 does not maintain mechanical hypersensitivity but does contribute to mechanistic understanding.

The interactions between ASIC3 and inflammatory cytokines are largely unknown. The data contained in Chapter IV demonstrate that inhibition of nuclear translocation of NF-κB prior to sensitization can block mechanical hypersensitivity, presumably by regulating the transcription of the inflammatory cytokines IL-1 and IL-6 that increase in muscle tissue after injection with acidified saline. Genetic manipulation of the IL-1 and IL-6 systems also prevents mechanical hypersensitivity after musculoskeletal sensitization. Prior studies demonstrate that pharmacological blockade of ASIC3 or genetic loss of the receptor prior to sensitization blocks mechanical hypersensitivity following musculoskeletal sensitization(310, 311, 329, 330). Although few studies have investigated the release of cytokines after activation of ASIC3 through acidosis, one *in vitro* study found increases in activated immune cells that release cytokines after acidosis in an ASIC3 dependent manner(313). An *in vivo* experiment using ASIC3 knockout mice with experimental arthritis detected increases in inflammation, especially IL-6, that was paradoxically associated with a reduction of pain(331). This limited data on the influence of ASIC3 activation on cytokine release requires further research to elucidate mechanisms of action.

ANIMAL RESEARCH

Animal models in biomedical research provide invaluable information on basic biology and pioneering treatments for diseases. Pre-clinical research is critical for performing studies that are too complex, costly, or dangerous to be performed with human subjects. The foundation of biological knowledge is derived from the results of studies that are invasive, intrusive, and potentially terminal. Pharmacological, surgical, and electrophysiological studies cannot generally use human subjects because of the unknown and potentially irreversible outcomes. For these reasons model organisms, including non-human primates, cats, dogs, rats, mice, fish, birds, and flies are used. Rodents, including rats and mice, are one of the most frequently used models in biomedical research. The sequencing of the mouse genome and creation of transgenic and knockout mouse lines has transformed the field of biomedical research. Yet the question still remains as to how translatable findings in rodents are for the reduction of disease burden in humans and contribution to public health. The role of rodent models within the fields of pain and sleep research are briefly discussed.

PAIN MODELS

Experimental pain models are cost-effective tools for the development and screening of analgesic treatments, however their translational value to humans is under scrutiny(159). Analgesics are the most prescribed pharmacological agents in the United States(332). Recently, several treatments developed using pre-clinical research techniques failed to translate successfully to human analgesia including the neurokinin 1 (substance P) receptor antagonists(333), sodium

channel blockers(334), and glycine-site antagonists(335). All of these interventions treated pain in pre-clinical trials only to fail early in human trials. These examples illustrate that despite drug safety and efficacy in animal models, there may be adverse side effects or a lack of analgesic benefit in humans(336). The absence of translational value from animal models in pain research has led to the suggestion that human subjects be utilized in the early stages of pain research(337). Although the use of human subjects is not without it's own ethical dilemmas, it may improve the success rate of treatment development. The breakdown between pre-clinical research and human testing poses a significant challenge to the treatment of pain and new opportunities need to be explored to develop successful treatments.

The challenges of animal research extend beyond the field of pain to immunology research(336). Recently, the translational value of sepsis research in rodent models is being called into question(338). The inflammatory pathways critical to the regulation and escalation of the inflammatory process during sepsis, a severe infection of the blood, are not the same in mice and humans(339). While this paper provides compelling data, it is also limited by only using one inbred mouse strain and does not reflect the genetic diversity of human sampling(338). These data challenge the field to examine the possibility of using alternate model systems, including pigs, which have an immune system more similar to humans(340). A global challenge like sepsis that requires an ongoing immune response may require an animal that more closely models

human physiology. The field of immunology provides one example of a breakdown between rodent research and the human condition.

The study of pain by its very nature requires the induction of physical discomfort that is not diminished through the use of analgesics. While this is not an insignificant undertaking ethically, the results are critical to understand biological mechanisms of pain. Pre-clinical models of pain focus on surgical manipulation, peripheral or systemic administration of exogenous agents, and pain as the result of disease states including cancer and diabetes(158). Research models cover a range of pain types including neuropathic, arthritic, immune, and cancer pain(158, 159). Substantial research is devoted to the study of neuropathic pain using surgical manipulation of the sciatic nerve(113, 321). Despite the contributions of these studies, neuropathic pain does not have the highest prevalence rates in the human population(4). Musculoskeletal pain contributes to three of the most prevalent pain conditions in the United States, represents one of the biggest barriers in returning to work after a workplace accident, and has some of the poorest patient reported rates of control(4, 100, 341). Investigations of the mechanisms of pain should consider epidemiological data and direct resources towards pain conditions prominent in public health. The implementation of better pre-clinical models of musculoskeletal pain, as well as more clinical research, may increase mechanistic understanding and novel translational approaches for musculoskeletal pain. Increasing mechanistic understanding and improving treatments of conditions involving musculoskeletal

pain will ultimately reduce the public health burden and improve patient quality of life.

The quantification of acute and ongoing pain in pre-clinical pain research is one of the significant challenges in the field. Research utilizes evoked and spontaneous behavioral tests prior to and following experimental manipulations to detect changes in pain(342). Pain can also be quantified between pre- and post-manipulation values, as well as placebo and experimental groups. However, the complex and diverse qualities of pain are not captured by these methods. The lexicon of terms describing pain in humans is rich with descriptors including burning, searing, tearing, throbbing, dull, aching, sharp, radiating, pressure, piercing, and tingling. Examining an animal that is hunched, demonstrating a weight bearing preference, or limping does not capture the qualitative nature of the pain. Similarly, tests of chemical, cold, heat, and pressure pain give a snapshot of an increase in responsiveness, reduction in threshold, or exacerbation of behavioral response, but not the burden of pain. This is difficult to extrapolate to chronic pain patients who execute day-to-day functioning in the face of tremendous discomfort. The development and validation of new techniques to measure pain in rodents is an area of progress for pre-clinical research. Unfortunately techniques requiring pre-training of animals to behaviorally respond and distinguish intensities of pain significantly slow the throughput on experiments and increase the costs of experiments(343, 344). The pre-clinical classification of pain is a critical factor in the translation of rodent research to the human condition.

SLEEP MODELS

The duration and distribution of sleep across the day of humans is different from that of rodents. Human sleep is consolidated to one sleep bout of approximately 8-hours during the dark period. In contrast, rodents are nocturnal polyphasic sleepers with shorter sleep bouts across the day with the largest consolidated sleep bouts occurring during the light period(345). One important difference between human and rodent sleep is the amount of time spent in REM sleep. Humans spend roughly 25% of the sleep period in REM sleep while rodents average between 7-10%(345). Despite the differences between human and rodent sleep there are many similarities, including the preservation of spectral characteristics of the EEG. Spectral similarities include the increase in high-amplitude EEG slow-wave activity during NREM sleep(345). Sleep responses to pathological conditions are also similar between the species. During acute infection humans and rodents increase NREM sleep that coincides with fever(346). Despite the differences between human and rodent sleep, laboratory mice and rats continue to be useful models for the study of sleep physiology.

Sleep deficits are a risk factor for pain, raising the question of whether enhancement of sleep can improve pain? Studies investigating the use of Zolpidem, a sleep aid, during the post-surgical recovery period of patients undergoing reconstructive knee surgery find that Zolpidem improves post-surgical pain(251, 252). Patients given Zolpidem as part of the medication regime during recovery report less subjective pain during the entire recovery period and

also require fewer analgesics to control their pain(251, 252). This finding is significant because opioids that are used to treat pain after surgical procedures are highly addictive, and can rapidly lose efficacy if taken in high doses(347). Opioids also contribute to the fragmentation of sleep, especially when taken for prolonged periods(257, 258). While data demonstrate the efficacy of Zolpidem in reducing pain burden after surgery, this should not bolster the market for somnogenic agents as a post-surgical medication. Non-pharmacological treatments have also been successfully used to treat sleep disorders and improve chronic pain. Cognitive behavioral therapy in patients with chronic pain, including musculoskeletal pain, improves subjective sleep quality and reduces pain(230, 231, 348). The use of pharmacological and behavioral treatments may contribute to a reduction in subjective pain, improvement in sleep quality, and decreased need for analgesic treatment in patients with chronic pain or undergoing painful procedures.

Adults, children, and adolescents all have declining sleep durations in the United States(3, 349). Cognitive behavioral therapy is one of the most successful treatments of insomnia(350), and is effective in improving sleep and pain symptoms in patients with chronic pain(351). Sleep is not a subject that is universally addressed in public education health classes, nor at higher levels of education(352). Given the pervasive nature of disordered sleep in the United States, education about establishing healthy sleep habits may curtail national sleep trends and improve the health of future generations(349, 353). Sleep is often overlooked until a precipitating event or injury, such as falling asleep while

driving. Although a simple technique, sleep education may reduce the number of persons voluntarily curtailing their sleep.

CONCLUDING REMARKS

Collectively, data presented in this dissertation contribute to our knowledge of the mechanistic interactions of musculoskeletal pain on sleep, the role of inflammatory cytokines on mechanical hypersensitivity, and demonstrate that SF combined with musculoskeletal sensitization exacerbates behavioral outcomes. The data indicate that musculoskeletal sensitization is a model useful in studying CNS interactions between pain and sleep. The burden of musculoskeletal pain in the general population encourages research into biological mechanisms of muscle pain and interactions with sleep. The physical, psychological, and emotional burden of pain, especially in conjunction with sleep disruption, tremendously impairs the daily functioning of patients. Research of the intersections between sleep and pain is necessary to develop effective treatments and reduce patient suffering associated with ongoing chronic pain and sleep disturbances.

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