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MALE-SPECIFIC MECHANISMS IN A MURINE MODEL OF NOCIPLASTIC PAIN

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MALE-SPECIFIC MECHANISMS IN A MURINE MODEL OF NOCIPLASTIC PAIN

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctorate of Philosophy

The University of Texas Medical Branch

March, 2022

Dedication

This dissertation is dedicated to my husband, Liam McDonough, who has unselfishly supported me through the challenges and joys of accomplishing this degree, as well as to our yet unborn child who has been with me while I completed my final experiments and wrote this dissertation. I love you both. *Deo Gratias*!

Acknowledgements

I would like to express my whole-hearted thanks to my mentors, Dr. Jun-Ho La and Dr. Jin Mo Chung, whose combined mentorship have made this work possible. They have not only provided me with fantastic scientific training but have also guided me with their wisdom and patience, challenging me to reach my fullest potential as a scientist. I also want to express my sincere gratitude to my supervisory committee members: Dr. Yogesh Wairkar, Dr. Jonathan Hommel, and Dr. Ru-Rong Ji for their guidance.

This work would not have been possible without the help of both past and current members of the Chung laboratory. I would especially like to thank Dr. Kali Hankerd, who was the co-first author on our published manuscript, as well Dr. Jigong Wang who performed the thermal sensitivity tests, and Jessica Tierney and Regan Hammond who contributed their efforts to the immunohistochemistry experiments in this project. I am also appreciative of Dr. Heidi Spratt who provided her valuable statistical consolation for this project, as well as Dr. Maxim Ivannikov and the UTMB Optical Microscopy Core for use of their facilities and troubleshooting assistance.

I am also grateful for the help and guidance I received from the Neuroscience Graduate Program, especially Debra Moncrief, Aurora Galvan, and Dr. Owen Hammill.

Finally, I would like to thank my family for their continuous love and support as I endeavored to complete this degree. To my parents, Lupita and William Winters, thank you for always encouraging me! To my husband, Liam, thank you for picking up the slack when I was overwhelmed with work, and for your endless encouragement. I also would like to thank Amber and Joe McDonough (as well as Joseph and Juliet) for allowing me to stay at their home the countless nights when I needed work late and did not want to make the long commute home. Your generosity will not be forgotten.

MALE-SPECIFIC MECHANISMS IN A MURINE MODEL OF NOCIPLASTIC PAIN

Publication No._____1_____

Kathleen Erin McDonough, Ph.D.

The University of Texas Medical Branch, 2022

Supervisors: Jun-Ho La & Jin Mo Chung

In order to address how acute nociceptive pain can transition to nociplastic pain, we developed a murine model in which acute injury-induced mechanical hypersensitivity was prolonged beyond its normal resolution time following postinjury stimulation at normally innocuous intensity. This model utilized intraplantar capsaicin injection or plantar incision as an acute injury, and vibration or warm water immersion for postinjury stimulation. The prolonged mechanical hypersensitivity in both males and females lasted at least 21 days in the absence of peripheral inflammation, indicating the nociplastic nature of this hypersensitivity. The persistent mechanical hypersensitivity was attenuated by morphine or gabapentin in both sexes but was maintained by sex-specific mechanisms: specifically, by ongoing peripheral afferent activity at the initial injury site in females and by reactive spinal microglia in males. Further investigation into the male-specific mechanisms underlying the nociplastic pain state revealed that activation of spinal microglia drives the postinjury vibration stimulation-triggered transition to a nociplastic pain state, but that microglia activation was not mediated by the BDNF-TrkB pathway, unlike other chronic pain models. After an acute peripheral injury, GABAergic disinhibition was required for postinjury vibration stimulation to trigger the spinal

microglia-driven transition to a nociplastic pain state. Even in the absence of an inciting peripheral injury, vibration stimulation could trigger the transition to a spinal microglia-mediated nociplastic pain state in males following direct spinal GABAergic disinhibition by intrathecal injection of the $GABA_A$ receptor antagonist bicuculline or the $GABA_B$ receptor antagonist CGP 52432. In females, spinal $GABA_B$ receptor inhibition, but not $GABA_A$ receptor inhibition, followed by vibration stimulation was able to trigger a transition to the nociplastic pain state. However, this pain state in females was not mediated by spinal microglia. Proinflammatory cytokines, but not prostaglandins, at the spinal level contributed to the maintenance of nociplastic pain state in males. Overall, these findings provide key insights for understanding the sex-specific mechanisms underlying the transition to and maintenance of the nociplastic pain state, indicating that spinal microglia are potential therapeutic targets to prevent and treat nociplastic pain in males.

TABLE OF CONTENTS

List of Figures

List of Abbreviations

BDNF	Brain-derived neurotrophic factor
BL	Baseline
COX	Cyclooxygenase
CRPS	Complex regional pain syndrome
GABA	Gamma-aminobutyric acid
GLMM	Generalized linear mixed model
IASP	International Association for the Study of Pain
Iba1	Ionized calcium binding adaptor molecule 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
KCC2	Potassium Chloride Cotransporter 2
LMM	Linear mixed model
LTMR	Low-threshold mechanoreceptor
mRNA	Messenger ribonucleic acid
NIH	National Institutes of Health
NSAID	Nonsteroidal anti-inflammatory drugs
sGABAn	Spinal GABAergic interneuron
TNF-α	Tumor necrosis factor alpha
TrkB	Tropomyosin receptor kinase B
UTMB	University of Texas Medical Branch
VFF	Von Frey Filament

Chapter 1: Introduction

BACKGROUND

Nociplastic Pain

There are three main categories of pain conditions: nociceptive, neuropathic, and nociplastic. *Nociceptive* pain occurs when there is threatened or actual damage to non-neuronal tissue and resultant inflammation following the injury. This pain is a normal, evolutionarily advantageous response to injury, as it both warns against potential danger and encourages the protection of an injured area to facilitate recovery. However, *neuropathic* pain, which is caused by damage in the somatosensory nervous system, and *nociplastic* pain, which is the subject of the present study, are conditions which are maladaptive chronic disorders severely impacting quality of life. Nociplastic pain is defined as pain arising from altered nociception despite lacking clear evidence of tissue injury activating peripheral nociceptors or somatosensory nerve damage (Kosek et al. 2016). In other words, changes within the undamaged pain circuitry itself abnormally produce chronic pain in nociplastic pain conditions. Nociplastic pain is seen in a multitude of chronic pain conditions including Complex Regional Pain Syndrome (CRPS) Type I, non-neuropathic chronic post-surgical pain, and fibromyalgia, among others, and may also be comorbid with other types of pain conditions. Often, nociplastic pain conditions present with additional non-pain symptoms including sleep disturbances, fatigue, and cognitive impairment (Fitzcharles et al. 2021).

Nociplastic pain is challenging to treat, as the underlying mechanisms are still poorly understood. Traditional analgesics such as nonsteroidal anti-inflammatory drugs

(NSAIDs) have been found to be less efficacious in nociplastic pain conditions (Derry et al. 2017), and opioid analgesics come with a number of adverse side effects, including the risk of addiction and opioid-induced hyperalgesia (Higgins, Smith, and Matthews 2019). Furthermore, due to lack of clear tissue or nerve damage, patient diagnosis can be delayed or symptoms doubted, leading to significant emotional distress. CRPS, for example, has an average time delay of nearly 4 years from onset of disease to diagnosis (Lunden, Kleggetveit, and Jørum 2016), and the average delay for fibromyalgia diagnosis has been reported as more than 6 years (Gendelman et al. 2018). On its own, this is clearly unacceptable, but combined with the fact that CRPS, and chronic pain conditions in general, are associated with higher rates of suicidal idealization and suicide attempts, this is nothing short of tragic (Sharma et al. 2009; Edwards et al. 2006). Therefore, it is of the utmost importance that nociplastic pain conditions be better studied to improve diagnosis and treatment outcomes for patients.

Central Sensitization and the Gate Control Theory of Pain

One key mechanism thought to underlie nociplastic pain is central sensitization. Central sensitization is characterized by heightened activity of nociceptive neurons in the central nervous system to afferent input. The perceptual/behavioral manifestation of central sensitization includes secondary mechanical allodynia, in which typically innocuous stimulation outside of any initial injury site abnormally produces pain (Sandkühler 2009; Latremoliere and Woolf 2009). A tissue injury can induce central sensitization that normally resolves as the injury heals. However, in the case of nociplastic pain conditions in which there is often an initial insult that has since fully

healed, the central sensitization induced by the initial insult may be maintained persistently, being disconnected from the status of the insult itself.

An understanding of the Gate Control Theory of Pain can help to explain, at least in part, how central sensitization occurs. In 1965, Drs. Ronald Melzack and Patrick Wall published the Gate Control Theory of Pain to explain the seemingly contradictory findings supporting the earlier Specificity and Intensity Theories of Pain. In this theory, primary afferents responsible for pain (nociceptors; C-fibers) and touch (mechanoreceptors; Aβ-fibers) form synapses in the dorsal horn both with spinal inhibitory (e.g., GABAergic) interneurons (sGABAn) and with projection neurons which send pain signals to the brain via the spinal anterolateral ascending tract. The sGABAn act as a "gate" within the dorsal horn to modulate the transmission of sensory information from peripheral afferents to nociceptive projection neurons. Specifically, Aβ-fibers activate sGABAn, which in turn inhibit the activation of projection neurons by C- and Aβ-fiber inputs, thus attenuating and/or preventing the transmission of pain signals to the brain. However, C-fibers inhibit sGABAn, effectively "opening the gate," allowing Aβ-fibers as well as those same C-fibers, to activate projection neurons (Melzack and Wall 1965; Moayedi and Davis 2012). Projection neurons are therefore abnormally activated by Aβ-fibers producing pain (allodynia) in response to stimuli which would normally encode light touch.

One important aspect of nociplastic pain, however, is that persistent mechanical allodynia is present despite lack of continuously activated nociceptors responsible for "opening the gate." In these cases, some other factors must be causing GABAergic disinhibition within the spinal cord. This disinhibition has been found to be produced by

a variety of factors, including changes within the GABAergic system itself, such as decreased synaptic excitation of GABAergic interneurons after intense nociceptor inputs (H. Y. Kim et al. 2015; Sivilotti and Woolf 1994), dysregulation of the Potassium Chloride Cotransporter 2 (KCC2) (K. Y. Lee and Prescott 2015), or as a result of Brain-derived neurotrophic factor (BDNF) modulation of $GABA_A$ receptor function (Chen et al. 2014). Other key factors known to play a role in disinhibition are proinflammatory cytokines. Proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, are produced in response to injury or inflammation, as well as by activated microglia (to be discussed in the next section). TNF-α has been shown to specifically decrease inhibitory synaptic transmission and inhibit spontaneous action potentials in sGABAn (H. Zhang, Nei, and Dougherty 2010). IL-1β has also been shown to increase overall dorsal horn excitability, due, at least in part, to inhibition of inhibitory tonic-firing neurons (Gustafson-Vickers et al. 2008). Likewise, soluble IL-6 was found to both inhibit GABA- and glycine-induced currents, as well as produce heat hyperalgesia (Kawasaki et al. 2008). Identifying which factors or combination of factors are underlying GABAergic disinhibition in different pain conditions is necessary for the proper treatment of individuals suffering from chronic pain disorders, as the mechanisms underlying central sensitization in chronic neuropathic or inflammatory pain may not be the same as in nociplastic pain.

Spinal Microglia and Pain

Microglia are the primary immune cells of the central nervous system, clearing cellular debris via phagocytosis and orchestrating inflammatory responses within the central nervous system (Lynch 2009). However, microglia can undergo a phenotypic transformation from “resting” to “activated”, which produces significant changes in both

their morphology and function. While in a “resting” state, microglia appear to have a small soma with numerous fine ramifications, which are used to monitor the central nervous system in order to detect changes in homeostasis. If such a threat to homeostasis is detected, such as inflammation or nerve damage, microglia undergo a rapid transformation process defined as microglia activation. As microglia become reactive, they appear amoeboid in shape, proliferate and migrate toward the detected threat, increasingly express certain cell surface markers such as Iba1, as well as release factors such as BDNF, proinflammatory cytokine, and chemokines. Furthermore, depending on the detected threat, microglia may take on distinct reactive phenotypes (Kettenmann et al. 2011; X. Zhang et al. 2014; Lynch 2009; D. Ito et al. 1998).

The changes associated with microglia activation are known to produce alteration in pain sensitivity (Gao and Ji 2010; Zhao et al. 2017). For example, proinflammatory cytokines such as IL-1β can increase the excitability of nociceptive neurons, contributing to pain hypersensitivity and central sensitization (Takeda et al. 2007; Gustafson-Vickers et al. 2008), and others have shown that neutralizing antibodies against TNF-α, IL-1β, and IL-6, all known to be released from spinal microglia, can attenuate mechanical allodynia after partial sciatic nerve ligation (Echeverry et al. 2017). Increased expression of chemokines and their receptors mediate neuronal signaling involved in chronic pain states (White and Wilson 2008; Clark and Malcangio 2014; Lindia et al. 2005). Furthermore, the involvement of microglia activation has been demonstrated in both chronic nerve injury and inflammatory pain models (Kohno et al. 2018; Svensson et al. 2003). Interestingly, the role of spinal microglia activation in central sensitization appears to be male-specific (Sorge et al. 2015; Taves et al. 2016). Understanding the sex-specific

mechanisms underlying chronic pain conditions, including nociplastic pain, is important for identifying sex-specific targets for the treatment of these conditions.

The mechanistic differences in regard to spinal microglia involvement in male but not female chronic pain models are neither fully understood nor consistent across the literature (Haight et al. 2020; Kwok et al. 2021; Koyanagi et al. 2016), but accumulating evidence points toward potential differences in male and female microglia themselves. A recent transcriptomic study revealed that in a murine models of chronic constriction injury, microglia from males and females showed differences in microglial gene expression (Fiore et al. 2022). Furthermore, others have found sex-specific features in murine microglia which have a basis in gestational hormone levels, both at the transcriptomic and phenotypic level. Male microglia were found to have a more inflammatory phenotype, while female microglia were neuroprotective, maintaining this distinct characteristic even when transplanted into male mice (Villa et al. 2018). It should be noted that this particular study was done in isolated brain microglia, and therefore whether spinal microglia also show these characteristics must still be investigated.

In addition to microglia's ability to release factors which alter pain sensitivity, nociceptors and GABAergic signaling have also been found to alter microglia function, indicating a potential place for microglia within the Gate Control Theory of Pain. For instance, in male rats, brief intense electrical stimulation of C-fibers was able to activate microglia in the dorsal horn, resulting in long-lasting mechanical hypersensitivity, demonstrating that afferent activity may directly activate microglia (Hathway et al. 2009). While this study only showed that stimulation of C-fibers, but not Aβ/δ-fibers,

could activate microglia, whether myelinated afferents could potentially activate microglia in pain states needs to be investigated.

In regard to microglial interactions with GABAergic signaling, it has been found that during early development, GABA-receptive microglia play a key role in pruning inhibitory synapses in a $GABA_B$ receptor-dependent manner (Favuzzi et al. 2021). Whether microglia continue to play any role in inhibitory pathway plasticity later in life upon injury has yet to be investigated, but could potentially shed light on microglia-dependent central sensitization. Beyond the developmental period, it has been reported that microglia express $GABA_B$ receptors, which upon activation, inhibit their proinflammatory function (Kuhn et al., 2004). Furthermore, cultured human microglia from brain tissue express mRNA and protein for GABA-transaminase, the enzyme which metabolizes GABA, and both $GABA_A$ and $GABA_B$ receptors. Furthermore, agonists of both $GABA_A$ and $GABA_B$ receptors were also found to attenuate cytokine release from these cultured human microglia (M. Lee, Schwab, and McGeer 2011). Further study into how the spinal GABAergic system may be able to regulate microglia, as well as reciprocal effects of microglia on GABAergic signaling are of great interest for the pain field.

SIGNIFICANCE

In to develop more efficacious and personalized treatments, the mechanisms underlying different types of chronic pain disorders, especially nociplastic pain disorders, must be better understood. Previously, there have been no animal models specific for the transition from acute to nociplastic pain conditions, producing an important gap in knowledge in terms of how an acute injury may trigger the development of a nociplastic pain state. With this question in mind, this project was developed in order to produce a novel murine model of “a transition to nociplastic pain” in order to investigate the sex-

specific mechanisms underlying the transition to and maintenance of the nociplastic pain state, with a specific interest in the role of microglia in nociplastic pain.

Building upon previous studies indicating that normally innocuous postinjury stimulation can prolong capsaicin-induced mechanical hypersensitivity (H. K. Kim et al. 2007; H. T. Kim et al. 2001), we hypothesized that 1) postinjury stimulation can induce a transition to a nociplastic pain state, which is maintained by sex-specific mechanisms, and 2) that spinal GABAergic disinhibition sex-dependently allows low-threshold mechanoreceptors to activate microglia, underlying the transition to nociplastic pain. Data gained from this research will help to identify key targets for sex-specific treatment and prevention of nociplastic pain.

Portions of Chapter 2 (Materials and Methods) as well as Chapter 3 (Postinjury stimulation triggers a transition to nociplastic pain in mice) in its entirety were previously published by PAIN® with a co-first authorship with Kali Hankerd (K. Hankerd et al. 2021).

Chapter 2: Materials and Methods

ANIMALS

Adult male and female C57BL/6N mice (aged 7-9 weeks) were purchased from Charles River (Houston, TX, USA) or bred inhouse. Mice were housed in groups of five in plastic cages with a 12-12 hour light-dark cycle and fed *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (UTMB) and in accordance with the National Institutes of Health (NIH) guidelines.

ANIMAL MODELS OF PAIN

Experimental Injury

All procedures were conducted while animals were under 1.5-2.5% isoflurane anesthesia.

INTRAPLANTAR CAPSAICIN INJECTION

As an experimental chemical injury, capsaicin (0.1% in 10% ethanol, 10% Tween-20, and 80% saline; Sigma-Aldrich, St. Louis, MO, USA) was injected intradermally at the (plantar side) base of the third and fourth digits of the left hind paw (3 µg in 3 µL) using a 30G needle.

PLANTAR INCISION

A group of mice underwent a plantar incision procedure instead of intraplantar capsaicin injection. The skin incision (~4.0 mm) was made along the (plantar side) base of the 2nd-4th digits and sutured (9-0 microsurgical needle; Fine Science Tools, Foster City, CA, USA).

Post-injury Stimulation

All procedures were conducted while animals were under 1.5-2.5% isoflurane anesthesia.

Post-injury thermal stimulation: In some groups of mice that received intraplantar capsaicin injection, the injured paw (i.e., the capsaicin injection area and surrounding tissues) was stimulated 2, 6, 24, or 72 hr after the injury. Post-injury thermal stimulation (30°C or 40°C) was applied in the following manner: the distal half of the hind paw (including the toes, injury area, and surrounding tissues, but not the von Frey testing site) was repeatedly submerged into temperature controlled sterile water for 30 sec with 30 sec intervals over a period of 10 min (10 times of 30 sec in and 30 sec out). When the experimenter's fingers were immersed in 40°C water, a sensation of warmth was elicited; no definitive cooling or warmth was sensed in 30°C water. In experiments when treatment timepoints coincided with behavioral tests, post-injury thermal stimulation was administered 30 min prior to behavioral tests. The timeline of these manipulations and behavioral testing is depicted in **Figure 1**.

As a control for the hind paw manipulation associated with water immersion after intraplantar capsaicin injection, a subset of mice was treated with capsaicin followed by "air immersion" 2 hr after capsaicin injection. Mice subjected to "air immersion" underwent the same dipping motion into an empty water-bath instrument.

In some mice that received plantar incision instead of capsaicin injection, 40°C post-injury thermal stimulation was performed 23 hr after the incision (i.e., 1 hr before von Frey assay at 24 hr post-incision) in the manner described above.

Post-injury vibratory stimulation: In experiments utilizing vibration (92 Hz) rather than water immersion, vibration was applied focally to the capsaicin-injected hind paw using a Hitachi HV-1 mini massager (2.25 mm^2 contact surface with 13-22 Pa of pressure). Vibration was applied for 10 sec with 30 sec intervals over 10 minutes. When tested on the experimenter's fingers, a mild but definite vibratory sensation was elicited. Sham vibration was used as a control, applying the vibration probe to the paw without turning on the vibrator.

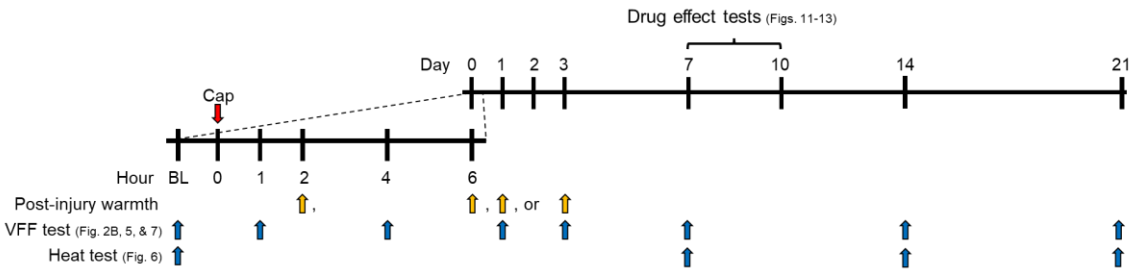

Figure 1: Timeline for behavioral experiments using capsaicin injection followed by postinjury thermal (warmth) stimulation.

After determining baseline (BL) behaviors, capsaicin (Cap, red arrow) was injected at the plantar side of the hind paw. The capsaicin-injected paw was immersed into warm water at time points marked by yellow arrows; at days 1 and 3 post-Cap, the warmth stimulation was delivered 30 minutes before von Frey filament (VFF) testing. VFF tests and radiant heat tests were conducted at predetermined times (blue arrows) in separate groups of mice. Effects of various drugs on persistent mechanical hypersensitivity were determined days 7 to 10 post-Cap.

Pain Models Lacking an Experimental Injury

Other experiments utilized nociplastic pain models lacking direct peripheral injury. Specifically, (+)-Bicuculline (30 ng/5 µL in 10% DMSO, 1% Tween-20 in saline; Sigma-Aldrich, St. Louis, MO, USA) or CGP 52432 (342.5 ng/5 µL in saline; Tocris, Bristol,

UK) was delivered intrathecally and followed 1 or 1.5 hour later by vibration stimulation as described above. Additionally, an animal model of intrathecal Brain-derived neurotrophic factor (BDNF) injection (Marcos et al. 2017; M'Dahoma et al. 2015; Zhou et al. 2011) was used, in which mice received a single intrathecal injection of human BDNF (10 ng/kg in saline, Alomone Labs, Jerusalem, Israel). All procedures were conducted while animals were under 1.5-2.5% isoflurane anesthesia.

BEHAVIORAL TESTS

Mechanical Sensitivity Test

Mice were habituated to behavioral test conditions (including experimenters) for four days prior to conducting behavioral procedures. Mice were placed into acrylic chambers (14 cm length x 5 cm width x 4.5 cm height) on a raised metal grid-floor platform and were acclimated to testing conditions for 30 minutes prior to testing on the day of experiment. Mechanical sensitivity of the capsaicin-injected hind paw was tested using a von Frey filament (0.98 mN) that evokes only 0-20% withdrawal responses in naïve mice. This mechanical force is below the mechanical thresholds of hind paw-innervating Aδ/C fibers, determined in ex-vivo skin nerve preparations from C57BL/6 mice (2.0-13.9 mN, (Milenkovic et al. 2008; Smith, O'Hara, and Stucky 2013) or in vivo from C3H/HeJ mice (the interquartile range 10-25 mN (Cain, Khasabov, and Simone 2001), and therefore unlikely to be a "threatened tissue damage causing the activation of peripheral nociceptors" in normal conditions. Considering the possibility that direct repeated probing of the injured area (e.g., capsaicin injection site) over time could be a confounding factor in experimentally defining post-injury stimulation, we stimulated the

area outside the injury (4-5 mm proximal to the injured area; mid-hind paw) with the 0.98 mN von Frey filament. Mechanical hypersensitivity is known to develop outside the injured area (commonly called secondary mechanical hypersensitivity) due to the injury-induced sensitization of nociceptive system at a central level (Melzack and Wall 1965; Sang et al. 1996; Simone et al. 1991; Torebjörk, Lundberg, and LaMotte 1992). The percent of withdrawal responses out of ten probing trials was recorded.

Thermal Sensitivity Test

After habituation as described above, mice were placed into acrylic chambers on a glass platform. A mobile laser emitter under the glass platform was placed beneath the middle of the hind paw and turned on. When the mouse withdrew the hind paw from the radiant heat of the laser, the emitter was automatically turned off and the latency to withdrawal was recorded. It should be noted here that the radiant heat could not be restricted to the outside of injured area, which therefore, could confound the "post-injury stimulation of injured area" paradigm when used before capsaicin-induced thermal hypersensitivity substantially abates. Therefore, except for the experiment determining the resolution time course of capsaicin-induced thermal hypersensitivity (i.e., Chapter 3, **Fig. 2A** and **Fig. 3**), we performed this radiant heat test at baseline and after persistent mechanical hypersensitivity was established.

DRUG ADMINISTRATION

For all injections, mice were anesthetized by 1.5-2.5% isoflurane, and injections were administered using a 30G needle. For experiments investigating the mechanisms of

transition from acute to nociplastic pain, mice received either a single intrathecal injection of (1) unconjugated saporin or Mac-1-saporin (8.85 µM, 5 µL; Advanced Targeting Systems, San Diego, CA) immediately after capsaicin injection or minocycline hydrochloride (150 µg, 5 µL, 10% DMSO, 0.5% Tween-20 in saline; Sigma-Aldrich, St. Louis, MO, USA) 1.5 hours post capsaicin; (2) recombinant human TrkB Fc chimera (2 µg, 5 µL, in saline; R&D Systems, Minneapolis, MN, USA) 1.5 hours post capsaicin or 30 minutes prior to intrathecal BDNF; or (3) muscimol (0.1 ug/ 5uL in saline; Tocris, Bristol, UK), or (+)-baclofen (0.06 ug/ 5uL in saline; Sigma-Aldrich, St. Louis, MO, USA) 1.5 hours post capsaicin.

For experiments studying the mechanisms of nociplastic pain maintenance, 7-10 days after the capsaicin injection, mice received 1) either a single intraplantar injection of 0.75% bupivacaine (3 µL; Sigma-Aldrich) or saline (0.9%; Baxter Healthcare Corporation, Deerfield, IL, USA) at the capsaicin injection area seven to ten days after capsaicin injection; 2) an intraperitoneal injection of morphine (5 mg/kg; Westward, Eatontown, NJ, USA), gabapentin (100 mg/kg; Spectrum Chemical Mfg Corporation, New Brunswick, NJ, USA) or saline (0.9%, Baxter Healthcare Corporation); 3) a single intrathecal injection of unconjugated saporin or Mac-1-saporin (8.85 µM, 5 µL; Advanced Targeting Systems, San Diego, CA, USA); (4) indomethacin (20 ug/ 5µL, 1% DMSO, 1% Tween-80 in saline; Sigma-Aldrich, St. Louis, MO, USA); (5) minocycline hydrocholoride (150 µg, 5 µL, 10% DMSO, 0.5% Tween-20 in saline; Sigma-Aldrich, St. Louis, MO, USA); or (6) a cocktail of neutralizing antibodies against TNF-α, IL-1β, and IL-6 (0.167 µg each anti-TNF-α, anti-IL-1β, anti-IL-6 in 5 µL of phosphate buffered saline, R&D Systems, Minneapolis, MN, USA) (Echeverry et al. 2017).

EVANS BLUE EXTRAVASATION

Under 2% isoflurane anesthesia, Evans Blue (50 mg/mL; Sigma Aldrich) was intravenously administered (50 mg/kg) via the tail vein either 2 hr, 1 day, or 7 days after capsaicin injection with or without 40ºC water immersion at 2 hr post-capsaicin; for 2 hr post-capsaicin time point data in the water immersion group, Evans Blue injection was done immediately after the immersion. Thirty minutes after Evans Blue injection, mice were perfused with saline, and glabrous skin samples (2 mm x 2 mm) from the capsaicin injection area and corresponding area on the contralateral hind paw were collected. Samples were dried in a 37°C oven for 72 hr. Evans Blue dye deposits were extracted in formamide (16 mL per 1.0 g dry weight tissue; Sigma Aldrich) at 37°C for 72 hr. The concentration of Evans Blue was quantified using a Nanodrop 2000C (Thermo Fisher, Waltham, MA, USA) and analyzed as described in the literature (Martin et al. 2010).

QUANTIFICATION OF PROINFLAMMATORY CYTOKINE GENE TRANSCRIPTS

On post-capsaicin injection day 1 or day 7, skin samples (2 mm x 2 mm) from the capsaicin injection area and corresponding area of the contralateral hind paw were collected and flash-frozen on dry ice. Skin tissue was diced finely, then transferred to an Eppendorf tube containing TRIzol reagent (Thermo Fisher) with 1 mg/mL collagenase type I (≥ 125 U/mg, Gibco, Waltham, MA, USA). Using a bead mill homogenizer with micro glass beads (0.5 mm, Biospecs Products, Bartlesville, OK, USA), tissue was processed for 10–15 cycles of 120 sec at speed 5. The supernatant was isolated and 400 µL pheno-chloroform (Amresco, Solon, OH, USA) per 1.0 mL TRIzol reagent was added. After centrifuging the mixture at 12,000 x g for 15 min, the supernatant was

transferred to a fresh Eppendorf tube containing an equal volume of isopropanol, incubated on wet ice for 10 min, and centrifuged at 10,000 x g for 10 min. Finally, the RNA pellet was rinsed three times with 1.0 mL 70% ethanol and centrifuged at 5,000 x g for 5 min prior to resuspension in nuclease-free water. RNA quality and purity were checked using a Nanodrop 2000C (Thermo Scientific) prior to being transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) per manufacturer instructions. SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was mixed with 10 ng of cDNA and β-2-microglobulin primer (forward: 5'-TGGTCTTTCTGGTGCTTGTC-3', 100 μM; reverse: 5'-GCAGTTCAGTATGTTCGGCT-3', 100 μM), IL-1β primer (forward: 5'-CTGGTGTGTGACGTTCCCATTA-3', 100 μM; reverse: 5'-CCACAGCACGAGGCTTT-3', 100 μM), IL-6 primer (forward: 5'-AAGAACAAAGCCAGAGTCCTTC-3', 300 μM; reverse: 5'-TAGGAGAGCATTGGAAATTGGG-3', 300 μM), or TNF-α primer (forward: 5'-CCCTCACACTCACAAACCAC-3', 300 μM; reverse: 5'-TTTGAGATCCATGCCGTTGG-3', 300 μM). qPCR was conducted at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 55.6°C for 30 sec, and 60°C for 30 sec. Single amplicon was confirmed by melt curve. As a readout of proinflammatory cytokine gene transcript amount, we first calculated a difference in quantification cycle number (ΔCq), detected using LinRegPCR software (Ramakers et al. 2003), between the reference gene (β-2-microglobulin) and the proinflammatory cytokine gene transcripts in each sample. Next, we calculated ΔΔCq by subtracting each ΔCq value from the mean of corresponding contralateral ΔCq values; the greater the ΔΔCq value, the greater the

amount of proinflammatory cytokine gene transcript in the ipsilateral side relative to that in the contralateral side.

IMMUNOHISTOCHEMISTRY

Twenty-four hours after treatment with intrathecal unconjugated saporin or Mac-1-saporin, or seven to ten days after capsaicin plus vibration, bicuculline plus vibration, or CGP 52342 plus vibration, mice were perfusion-fixed using phosphate buffered saline (PBS) followed by Lana's fixative (4% paraformaldehyde and 14% picric acid in PBS). The lumbar spinal cord (L3–L5) was resected and post-fixed in Lana's fixative for 4 hr, and then dehydrated in 30% sucrose solution overnight. Samples were then embedded in Tissue-Plus Optimal Cutting Temperature (O.C.T.) Compound (Fisher Health Care, Norwich, UK). Lumbar spinal cord sections (12 µm thick) were stained for Iba1 using an anti-Iba1 primary antibody (1:2,000, rabbit; Wako, Japan), followed by AlexaFluor 488-conjugated anti-rabbit secondary antibody (1:300; Thermo Fisher). Stained sections were mounted with DAPI-containing media (Vectashield; Vector Laboratories, Burlingame, CA, USA). ImageJ was used to analyze confocal microscope images by percent area florescence (La et al. 2016; F. Zhang et al. 2012) within the medial dorsal horn.

STATISTICAL ANALYSES

In experiments assessing thermal sensitivity by measuring paw withdrawal latency at multiple time points, mean±SD or individual values are presented, and data were analyzed using linear mixed model (LMM) with the first order autoregressive (AR1)

covariance structure for repeated measures (Time) followed by Sidak multiple comparison tests. To delineate the resolution time course of capsaicin-induced thermal hypersensitivity, the difference between withdrawal latencies at baseline and each time point (ΔL_t) was normalized to the peak difference at 2 hr post-capsaicin (ΔL_{2h}) in individual mice. Curve fitting was performed using a single-phase exponential decay function in each sex and statistically tested to determine whether different curves fit for male and female data (Prism ver. 8, GraphPad, CA, USA).

In experiments assessing mechanical sensitivity by counting the number of paw withdrawals out of 10 trials at multiple experimental time points, data are presented as mean with standard error of the mean (SEM) and were analyzed using generalized linear mixed model (GLMM) with a logit link function for binomial distribution and AR1 covariance structure for repeated measures; sequential Sidak procedure was used for multiple comparisons between groups at each time point; degrees of freedom were allowed to vary across tests (SPSS ver. 25, IBM, NY, USA).

In an Evans Blue extravasation assay, data were log-transformed to resolve heteroscedasticity and analyzed using LMM (Paw side [nested within an animal] x Model x Time in each sex; Bonferroni test for multiple comparisons). The levels of gene expression were analyzed by comparing $\Delta\Delta$Cq value between groups using LMM (Paw side [nested within an animal] x Model x Time in each sex; Bonferroni test for multiple comparisons).

Iba1-immunoreactive density values were obtained from each dorsal horn side of multiple sections from a single mouse. A multilevel analysis (linear mixed model) was used for this nested design (two dorsal horn sides nested within a section and multiple

sections nested within a mouse) with random intercepts. We took an *a priori* approach for predetermining comparison pairs by Bonferroni tests (e.g., ipsilateral vs. contralateral sides in each treatment); P value was adjusted.

Chapter 3: Post-injury stimulation triggers a transition to nociplastic pain in mice[1]

INTRODUCTION

Tissue injury produces nociceptive pain that gradually subsides as the injury heals and local inflammation resolves. However, as in chronic pain conditions such as complex regional pain syndrome (CRPS) type I and chronic post-surgical pain, pain can persist long after the inciting injury has healed, without apparent organic abnormalities underlying the pain (McCabe and Blake 2008; Steegers et al. 2008). This type of chronic pain, recently termed "nociplastic pain" by the International Association for the Study of Pain (IASP) taskforce, is a unique category of chronic pain disorders distinct from idiopathic and neuropathic pain (Kosek et al. 2016). In such disorders, nociplastic pain originates from "altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system" (Kosek et al. 2016). Owing to its heterogeneous etiology and a lack of mechanistic insight, the development and implementation of effective therapeutics to prevent and treat nociplastic pain conditions have been greatly hindered.

There are multiple critical questions which must be addressed in nociplastic pain studies. Notably, females are disproportionately affected by nociplastic pain conditions (Kehlet, Jensen, and Woolf 2006; Melchior et al. 2016; Sandroni et al. 2003). The female

[1] This chapter was published by PAIN. Hankerd K, McDonough KE, Wang J, Tang SJ, Chung JM, La JH. Postinjury stimulation triggers a transition to nociplastic pain in mice. PAIN. 2022 March 1. doi: 10.1097/j.pain.0000000000002366. PMID: 34285154.

overrepresentation in chronic pain conditions in general has been attributed to both biological and psychosocial factors. However, the potential contribution of inherent female susceptibility or sexually dimorphic pain mechanisms to the incidence of nociplastic pain is unclear. Aside from sex, it is noteworthy that in some nociplastic pain conditions such as CRPS type I ("Complex Regional Pain Syndrome Fact Sheet" 2020), fibromyalgia (Dadabhoy and Clauw 2006), post-infectious irritable bowel syndrome, and chronic post-surgical pain, potential or obvious inciting injuries (trauma, infection, etc.) could be identified. Since such injuries do not always result in nociplastic pain conditions, it is of interest and significance to understand how the injury-induced initial pain transitions to nociplastic pain, and how the nociplastic pain state is maintained, once established, in the absence of ongoing tissue damage.

With respect to such transition, chronic post-surgical pain provides valuable information about risk factors. Surgery being considered a tissue injury by itself or a post injury insult, clinical findings indicate that the magnitude of pain after or before surgery may be predictive of its "chronification" (Yarnitsky et al. 2008). Designing experimental approaches for modeling nociplastic pain in animals to address the abovementioned questions, we used these clinical findings to experimentally trigger the transition from an injury-induced, normally resolving pain to nociplastic pain in an animal model of acute injury. Specifically, we enhanced post-injury pain by stimulating the injured area. Our previous studies show that capsaicin-induced mechanical hypersensitivity in rats is transiently enhanced and prolonged by stimulation of the capsaicin-injected paw even at a normally innocuous intensity (H. K. Kim et al. 2007; H. T. Kim et al. 2001). Using this paradigm, we established here that mechanical hypersensitivity can be significantly

prolonged after post-injury stimulation in both male and female mice, without apparent persistent tissue damage, and that this prolonged mechanical hypersensitivity is maintained by sexually dimorphic mechanisms. Portions of these studies have been reported in abstract form (K. M. Hankerd, La, and Chung 2019; K. E. McDonough et al. 2019).

RESULTS

Intraplantar capsaicin produced pain hypersensitivity that resolved in different time courses between sexes

Our model utilizes 0.1% capsaicin injection as an experimental injury, followed by post-injury thermal stimulation. This concentration of capsaicin is commonly used as a chemical injury for producing acute pain by directly activating nociceptors (primarily producing a burning sensation) (Schmelz et al. 2000) and inducing both peripheral (Baumann et al. 1991) and central sensitization (Gazerani, Andersen, and Arendt-Nielsen 2005; La et al. 2017). Prior to developing a nociplastic pain animal model using post-injury thermal stimulation to increase post-injury pain, we first characterized capsaicin-induced sensitization, focusing on sex differences in the magnitude of capsaicin-induced thermal and mechanical hypersensitivity. In our radiant heat test condition, males showed a longer latency to withdrawal at baseline and a steeper trajectory back to the baseline level after capsaicin injection (**Figure 2A**). Curve fitting of the resolution time course of capsaicin-induced thermal hypersensitivity (with the hypersensitivity at 2 hr post-capsaicin being set as the 100% peak for each mouse) revealed slower resolution in females than in males (**Fig. 3**: R^2=0.73 for females, 0.84 for males; F(2,90)=24.8,

p<0.001). A resolution time constant (tau) was 3.1 days for females (95% CI: 2.3-4.1 days) and 1.0 day for males (95% CI: 0.8-1.2 days), suggesting a slower subsidence of capsaicin-induced thermal nociception in females.

We also noted differences in the resolution of capsaicin-induced mechanical hypersensitivity. While both sexes showed a similar degree of mechanical hypersensitivity at 1 hr post-capsaicin and a gradual decrease over the following 3 days, females manifested significantly greater mechanical hypersensitivity than males at 1 day post-capsaicin (**Fig. 2B**: t(105)=2.60 by sequential Sidak test, p=0.011).

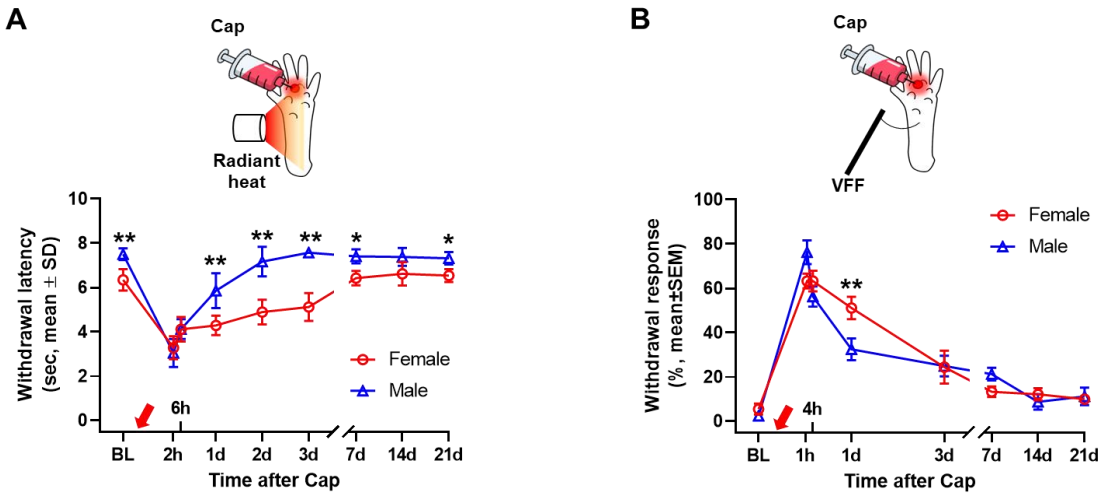

Figure 2: Sex difference in capsaicin-induced thermal and mechanical hypersensitivity.

(A) Mice developed thermal hypersensitivity after intraplantar capsaicin (Cap, red arrow) injection, showing decreased latency to withdraw from radiant heat. Females (n=6) showed a slower recovery to the baseline (BL) level than males (n=8 until day 1, n=6 on day 2, n=4 in days 3-21). (B) Mice developed mechanical hypersensitivity after intraplantar Cap injection, showing increased hind paw withdrawals from von Frey filament (VFF, 0.98 mN) probing of an area outside the Cap injection site, which gradually resolved in 7 days. Females (n=9) showed greater mechanical hypersensitivity at 1 day post-capsaicin than males (n=8). *p<0.05, **p<0.01 between males and females by the Sidak multiple comparison test.

We next determined whether the movement of the capsaicin-injected hind paw for post-injury thermal stimulation itself would affect capsaicin-induced mechanical hypersensitivity in the area outside the capsaicin injection site. This "air immersion" was done 2 hr after capsaicin injection. As shown in **Fig. 4A and B**, the magnitude of mechanical hypersensitivity was not significantly different between the capsaicin alone and the "capsaicin plus air dip" groups in each sex (Cap vs. Cap+air dip across the experimental time points: $F(1,27)=0.33$, $p=0.57$ in females; $F(1,13)=0.051$, $p=0.82$ in males by GLMM analysis). As the results indicated that the hind paw immersing movement itself does not change capsaicin-induced mechanical hypersensitivity, we pooled the "capsaicin plus air immersion" group with the "capsaicin alone" group and regarded them as "capsaicin controls" throughout this study.

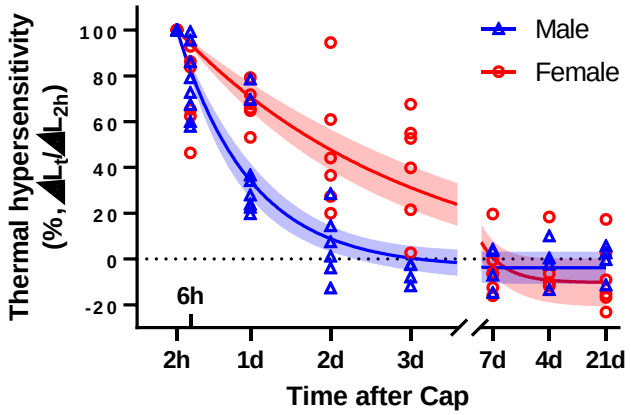

Figure 3: *Curve fitting of capsaicin-induced thermal hypersensitivity revealed slower resolution of the hypersensitivity in females than in males.*

ΔLt, the difference between withdrawal latencies at baseline and each time point; ΔL2h, the peak difference (100%) at 2 hr post-Cap. The shaded area indicates 95% confidence interval. Males: n=8 until day 1, n=6 on day 2, n=4 in days 3-21. Females: n=6 throughout.

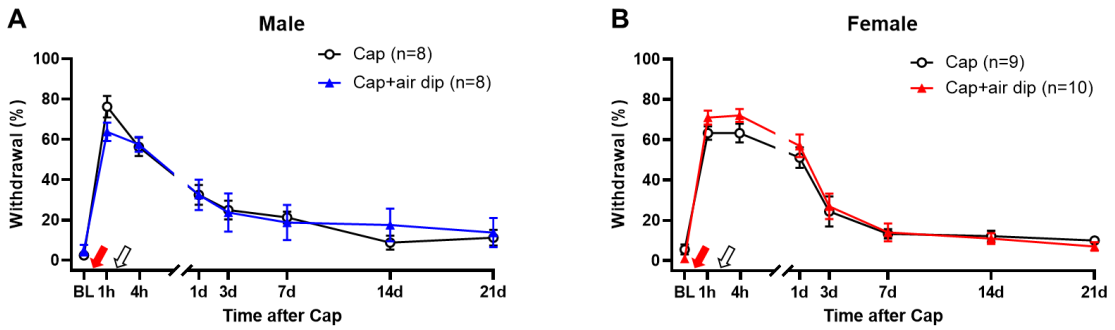

Figure 4: Capsaicin-induced mechanical hypersensitivity is not affected by hind paw immersing motion itself.

In both (A) males and (B) females, the dipping motion (white arrow) of the capsaicin (Cap, red arrow)-injected hind paw into an empty water bath does not affect the magnitude and time course of Cap-induced mechanical hypersensitivity. Data are presented as mean±SEM. Males: n=8 in Cap and n=8 in Cap+air dip. Female: n=9 in Cap and n=10 in Cap+air dip. BL, baseline.

Post-injury stimulation prolonged injury-induced mechanical hypersensitivity

Having determined that the hind paw immersing movement itself does not affect capsaicin-induced mechanical hypersensitivity, we next assessed whether the hypersensitivity could be prolonged by stimulating the capsaicin-injected paw with 40°C water immersion (30 sec per min for 10 min at 2 hr after capsaicin; the von Frey testing site was not immersed). This temperature is normally innocuous but reported to cause discomfort in humans after development of capsaicin-induced thermal hypersensitivity (Moulton et al. 2007). While the 40°C stimulation of the vehicle-injected hind paw did not induce mechanical hypersensitivity (data not shown), this thermal stimulation applied to the capsaicin-injected hind paw significantly prolonged capsaicin-induced mechanical hypersensitivity (**Fig. 5**); capsaicin plus 40°C group showed greater mechanical hypersensitivity than capsaicin control in both sexes from day 1 (male) or day 3 (female)

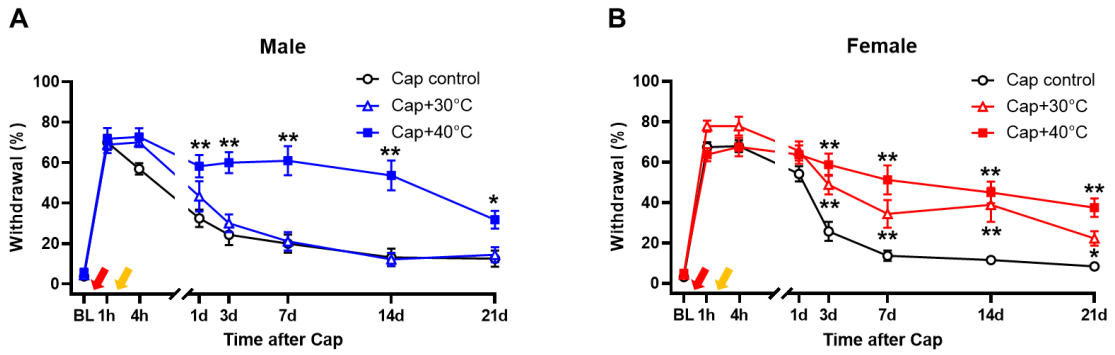

Figure 5: Chronification of capsaicin-induced mechanical hypersensitivity by postinjury thermal stimulation.

In both males (A) and females (B), stimulation of capsaicin (Cap, red arrow)-injected area by 40°C water (yellow arrow) at 2 hours post-capsaicin significantly prolonged capsaicin-induced mechanical hypersensitivity. However, when 30°C water was used instead of 40°C water, only females showed a significant chronification. Males: n=16 in Cap control, n=9 in Cap+30°C, and n=11 in Cap+40°C. Females: n=19 in Cap control, n=9 in Cap+30°C, and n=8 in Cap+40°C. *p<0.05, **p<0.01 vs Cap control by sequential Sidak multiple comparison tests; as the same Cap control and Cap+40°C (at 2 hours) groups were used for statistical comparisons in Fig. 7, differences between Cap control and all the other groups in Figures 5 and 7 were analyzed in the same statistical test. BL, baseline.

and on up to day 21 post-capsaicin. This prolonged mechanical hypersensitivity was no longer present by day 28 in both sexes (males: median=10%, IQR=0-10%, n=9; females: median=10%, IQR=0-17.5%, n=8). We next asked whether chronification of capsaicin-induced mechanical hypersensitivity would depend on the intensity of post-injury thermal stimulation. When the capsaicin-injected hind paw was stimulated with 30°C water at 2 hr post-capsaicin, capsaicin-induced mechanical hypersensitivity was still significantly prolonged in females, but not in males. Based on the results that 40°C water immersion reliably prolongs capsaicin-induced mechanical hypersensitivity in both sexes, we chose this as the intensity of post-injury thermal stimulation for all future experiments. Of note, capsaicin-induced thermal hypersensitivity was not prolonged by the 40°C post-injury thermal stimulation; the latency to withdraw from radiant heat at 7-21 days post-capsaicin

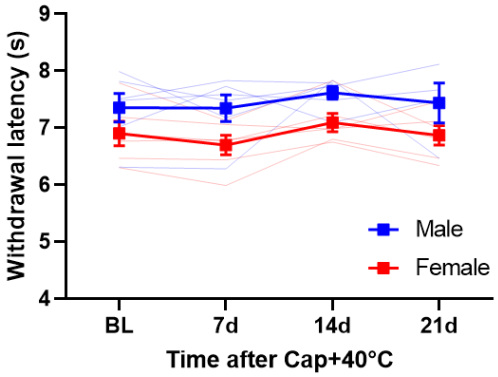

Figure 6: Chronification of capsaicin-induced thermal hypersensitivity does not occur by 40°C postinjury stimulation.

In both sexes, the heat sensitivity, measured as the latency to withdraw from radiant heat, did not differ between baseline (BL) and 7 to 21 days after the stimulation of capsaicin (Cap)-injected area with 40°C water at 2 hours post-capsaicin. Males: n=6 until day 14 and n= 4 at day 21. Females, n=6 throughout. Thick lines with square symbols indicate mean±SEM of each sex. Thin lines indicate individual animals.

did not differ from the baseline values in both sexes (**Fig. 6**). Therefore, we focused our later studies on chronification of mechanical hypersensitivity.

In our modeling approach, post-injury stimulation was used to increase pain after injury, based on clinical findings indicating that greater pain after surgery (i.e., surgery being an injury) increases the risk of chronification of post-surgical (i.e., post-injury) pain. Therefore, in our experimental design using capsaicin as an experimental injury, we hypothesized that the effect of post-injury 40°C stimulation, which serves to increase post-injury pain, will diminish as the injury-induced thermal hypersensitivity abates, consequently reducing the likelihood of mechanical hypersensitivity chronification. Thus, we next tested whether the 40°C post-injury stimulation would still effectively induce the chronification of mechanical hypersensitivity when the capsaicin-induced thermal hypersensitivity decreased to approximately 70% or 35% of the peak thermal hypersensitivity (100% at 2 hr post-capsaicin). Because the thermal hypersensitivity resolves differentially between sexes as shown in **Fig. 3**, we chose sex-specific time points for the two abating phases: 6 hr vs. 1

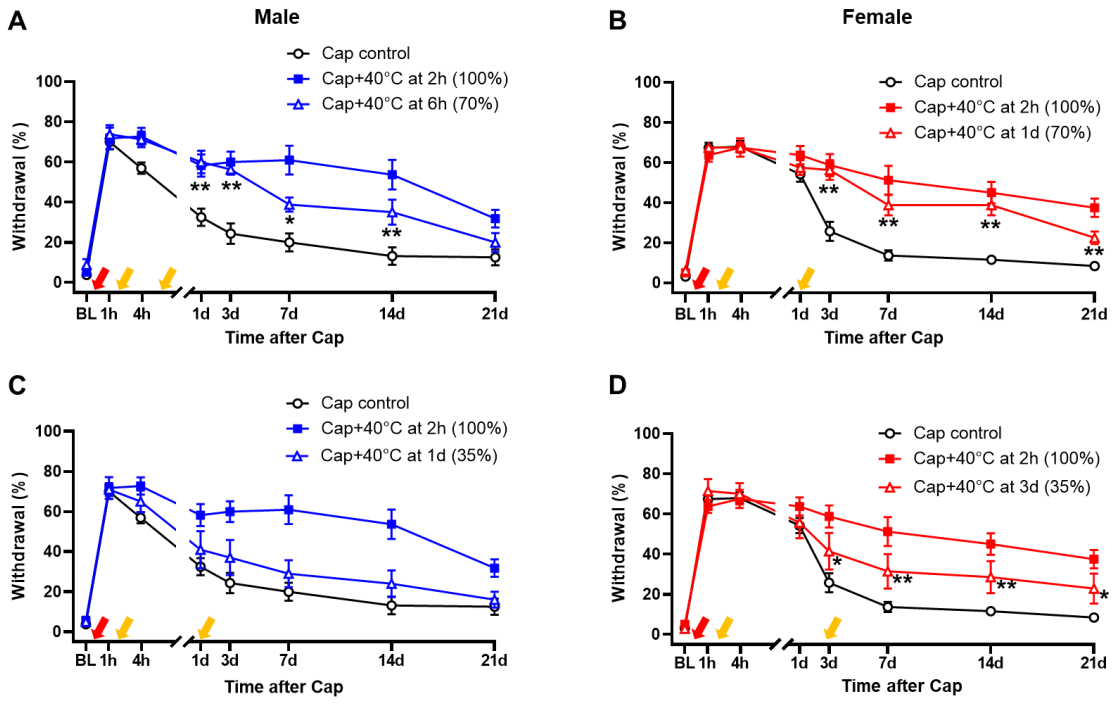

Figure 7: *Magnitude of capsaicin-induced thermal hypersensitivity at the time of postinjury thermal stimulation is predictive of mechanical hypersensitivity chronification.*

In both males (A) and females (B), mechanical hypersensitivity was significantly prolonged by the postinjury thermal stimulation (yellow arrow) when capsaicin (Cap, red arrow)-induced thermal hypersensitivity has abated to ~70% of the peak (6 hours in males and 1 day in females). However, when the thermal hypersensitivity decreased to ~35% of the peak (1 day in males and 3 day in females), the 40°C postinjury stimulation did not produce chronification of capsaicin-induced mechanical hypersensitivity in males (C); females still showed significantly greater mechanical hypersensitivity at time points ≥ day 3 post-Cap (D). Males: n=16 in Cap control, n=11 in Cap+40°C at 2 hours, n=8 in Cap+40°C at 6 hours, and n=10 in Cap+40°C at 1d. Females: n=19 in Cap control, n=8 in Cap+40°C at 2 hours, n=8 in Cap+40°C at 1d, and n=7 in Cap+40°C at 3d. *p<0.05, **p<0.01 vs Cap control by sequential Sidak multiple comparison tests; as the same Cap control and Cap+40°C at 2 hours groups were used for statistical comparisons in Fig. 5, differences between Cap control and all the other groups in Figures 5 and 7 were analyzed in the same statistical test. BL, baseline.

day post-capsaicin for males and 1 day vs. 3 days post-capsaicin for females to represent the 70% vs. 35%, respectively based on curve fitting results. In mice that receive this "delayed" 40°C stimulation, no difference vs. capsaicin control would be expected prior

to the post-injury stimulation. When capsaicin-induced thermal hypersensitivity abated to approximately 70% (i.e., 6 hr in males and 1 day in females), the 40°C stimulation still significantly increased mechanical hypersensitivity at time points later than the time point of 40°C stimulation (**Fig. 7A and B**). When the thermal hypersensitivity subsided to approximately 35% (i.e., 1 day in males and 3 days in females), mechanical hypersensitivity was not prolonged in males (**Fig. 7C**); however, in females, the hypersensitivity was still significantly increased at time points later than day 3 (**Fig. 7D**; note that the post-injury stimulation was applied 30 min before the behavioral tests on day 3). These data suggest that the magnitude of capsaicin-induced thermal hypersensitivity at the time of post-injury thermal stimulation is predictive of mechanical hypersensitivity chronification and females have a wider timeframe than males, in which post-injury stimulation can trigger such chronification.

Next, we determined whether a different type of post-injury stimulation would also be able to prolong mechanical hypersensitivity. When vibration (92 Hz, 10 sec at 30 sec intervals over 10 min) was used instead of thermal stimulation at 2 hr post-capsaicin, mechanical hypersensitivity was effectively prolonged as shown in **Fig. 8A and B**. This result suggested that chronification of mechanical hypersensitivity may be induced by different modalities of post-injury stimulation.

We next investigated the potential limitation of the ability for post-injury thermal stimulation to prolong mechanical hypersensitivity in other types of injuries. In this experiment, we applied the 40°C thermal stimulation to an incision injury area at 23 hr post-incision (i.e., 1 hr before behavioral tests on day 1). We found that the incision-induced mechanical hypersensitivity was significantly prolonged as shown in **Fig. 8C**

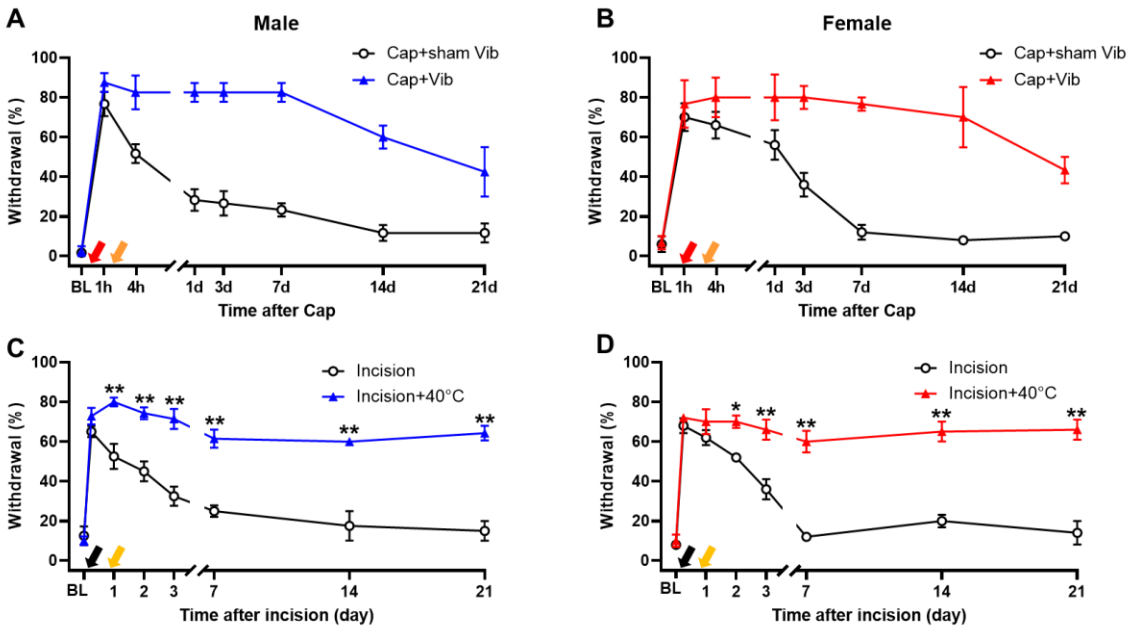

Figure 8: Confirmation of mechanical hypersensitivity chronification using different modeling paradigms.

(A and B) When the capsaicin (Cap)-injected hind paw was stimulated with vibration (92 Hz, 10 seconds at 30-second intervals for 10 minutes, orange arrow) at 2 hours post-capsaicin, Cap-induced mechanical hypersensitivity was significantly prolonged in both sexes. The vibrator probe was placed on the paw but not turned on in Cap+sham Vib group. In both males and females, n=5 in each group. (C and D) When the hind paw was immersed in 40°C water (30 seconds/minute for 10 minutes, yellow arrow) 23 hours after plantar incision, the incision-induced mechanical hypersensitivity was significantly prolonged in both sexes. Males: n=4 in incision and n=7 in incision+40°C. Females: n=5 in each group. $^{**}p<0.01$ vs corresponding control. BL, baseline. Vib, vibration.

and D. This data indicated that mechanical hypersensitivity chronification by post-injury thermal stimulation is not restricted to the capsaicin model. Observing that these different modeling paradigms commonly demonstrate that post-injury stimulation prolongs post-injury mechanical hypersensitivity beyond the normal resolution time, we chose to use capsaicin injection followed by 40°C thermal post-injury stimulation at 2 hr (capsaicin plus 40°C) throughout the rest of this study.

No clear evidence of persistent inflammation at the previous injury area to account for the persistent mechanical hypersensitivity

Having found that post-injury stimulation of the capsaicin-injected paw at 2 hr post-capsaicin made the capsaicin-induced mechanical hypersensitivity persistent, we next examined whether this persistent hypersensitivity could be accounted for by persistent inflammation at the capsaicin-injected paw, as capsaicin produces neurogenic inflammation (Jancsó, Jancsó-Gábor, and Szolcsányi 1968; Q. Lin, Wu, and Willis 1999). Visual examination of the footpad during our experiments did not reveal obvious tissue damage such as skin discoloration or edema. As inflammation may be present without

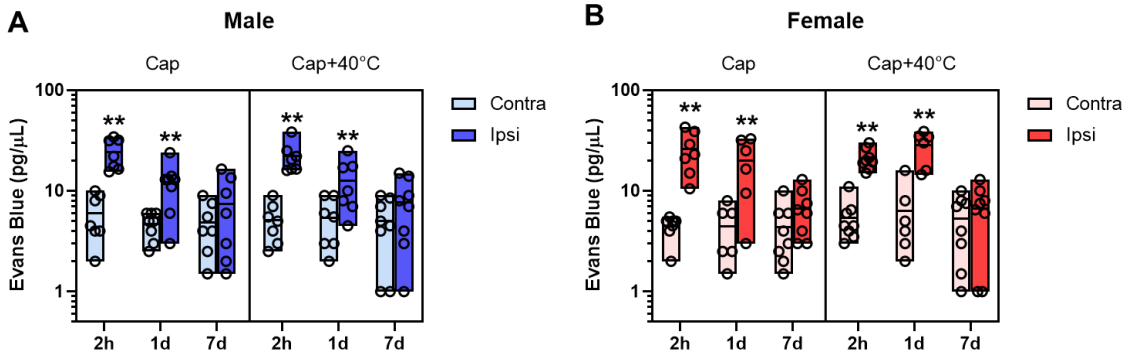

Figure 9: No evidence of increased vascular leakage at the capsaicin-injected area 7 days after capsaicin plus 40°C postinjury stimulation.

In both males (A) and females (B), Evans Blue extravasation, an indicator of vascular leakage associated with tissue inflammation, was significantly greater in the capsaicin (Cap)-injected area than in the corresponding contralateral area at 2 hours and 1 day post-capsaicin. However, such increased vascular leakage was not detected 7 days after Cap or Cap plus 40°C postinjury stimulation (Cap+40°C). ** $p<0.01$ between paired paw sides by Bonferroni tests for 9 pairwise comparisons. The bottom, middle line, and top of each bar indicate the minimum, mean, and maximum values in each group, respectively. Males: at 2 hours, n=7 in Cap and in Cap+40°C; at 24 hours, n= 8 in Cap and n=7 in Cap+40°C; at day 7, n=7 in Cap and n= 8 in Cap+40°C. Females: at 2 hours, n=7 in Cap and n=8 in Cap+40°C; at 24 hours, n=6 in Cap and Cap +40°C; at day 7, n=8 in Cap and Cap+40°C.

overt abnormalities, we next used an Evans Blue extravasation assay to assess whether plasma extravasation, an indicator of inflammation, was augmented at the capsaicin injection area (Martin et al. 2010). Two hours after capsaicin injection, plasma extravasation was significantly increased at the injection area compared to the corresponding contralateral area (**Fig. 9**: $t(38)=6.66$, $p<0.001$ in males; $t(37)=8.43$, $p<0.001$ in females by Bonferroni test for 9 pairwise comparisons). A similar increase was detected in the capsaicin plus 40°C group at 2 hr post-capsaicin ($t(38)=6.73$, $p<0.001$ in males; $t(37)=7.52$, $p<0.001$ in females). Plasma extravasation at the capsaicin injection area was still increased in both sexes at day 1 in capsaicin control ($t(38)=4.09$, $p<0.005$ in males; $t(37)=6.45$, $p<0.001$ in females) and capsaicin plus 40°C groups ($t(38)=3.82$, $p=0.004$ in males; $t(37)=7.62$, $p<0.001$ in females). However, at day 7, plasma extravasation was not significantly different from that of the corresponding contralateral area in all groups. These data suggested that capsaicin-induced local inflammation has resolved by day 7 in both capsaicin control and capsaicin plus 40°C group.

In addition, we quantified proinflammatory cytokine gene transcripts at the capsaicin injection area. As shown in **Fig. 10**, tissue contents of IL-1β mRNA in the capsaicin injection area were greater in the capsaicin control ($t(18.5)=4.20$, $p=0.003$ in males; $t(16.7)=2.84$, $p=0.07$ in females by Bonferroni test for 6 pairwise comparisons) and capsaicin plus 40°C group ($t(7.8)=4.48$, $p=0.017$ in males; $t(6.43)=5.0$, $p=0.015$ in females) than in corresponding contralateral areas at 1 day post-capsaicin. At this time point, gene expression of other proinflammatory cytokines were also elevated in the capsaicin injected area. TNF-α mRNA level was high in male capsaicin control ($t(26)=2.77$, $p=0.06$) and female capsaicin plus 40°C groups ($t(10.3)=5.15$, $p=0.003$). Of

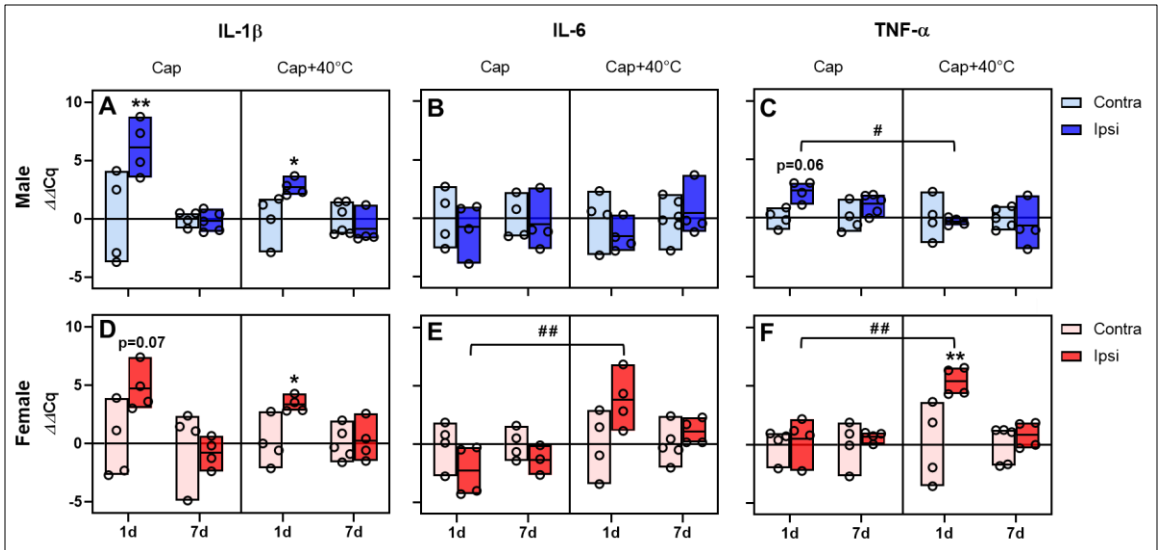

Figure 10: No evidence of increased gene expression of proinflammatory cytokines at the capsaicin-injected area 7 days after capsaicin plus 40°C postinjury stimulation.

In both males (A) and females (D), the quantity of interleukin (IL)-1β gene transcript was greater in capsaicin (Cap)-injected area (Ipsi) than in the contralateral counterparts (Contra) at 24 hours post-capsaicin. However, such upregulation was not detected 7 days after Cap or Cap plus 40°C postinjury stimulation (Cap+40°C). On day 7, the gene expression of IL-6 (B and E) and tumor necrosis factor (TNF)-α (C and F) also did not differ between contralateral and ipsilateral sides. Of note, the TNF-α gene expression in the ipsilateral side (C) was significantly lower at 24 hours post-capsaicin in the male Cap+40°C group, compared with that in Cap control. In female Cap+40°C group, IL-6 (E) and TNF-α (F) mRNAs were significantly increased in the ipsilateral side at 24 hours post-capsaicin, compared with those in female Cap control. *p<0.05 and **p<0.01 between paw sides; #p<0.05 and ##p< 0.01 between Cap and Cap+40°C groups in the ipsilateral side by Bonferroni tests for 6 pairwise comparisons. The bottom, middle line, and top of each bar indicate the minimum, mean, and maximum values in each group, respectively. Males: in IL-1β, n=4 in contra and n=5 in ipsi for Cap; n=6 in contra and n=4 in ipsi for Cap+40°C; in IL-6, n=4 in contra and n=5 in ipsi for Cap; n=5 in contra and n=4 in ipsi for Cap+40°C; for TNF-α, n=4 in contra and n=5 in ipsi for Cap; n=5 in contra and n=4 in ipsi for Cap+40°C. Females: in IL-1β, n=4 in both sides for Cap; n=5 in contra and n=4 in ipsi for Cap +40°C; in IL-6, n=4 in both sides for Cap; n=5 in contra and n=4 in ipsi for Cap+40°C; for TNF-α, n=4 in both sides for Cap; n=5 in contra and n=4 in ipsi for Cap+40°C.

note, statistically significant increases in the quantities of IL-6 (t(23.9)=4.44, p=0.001) and TNF-α mRNAs (t(23.7)=3.82, p=0.005) were detected only in female capsaicin plus

40°C group, compared with their capsaicin control counterparts.

In line with the above plasma extravasation results, the quantities of these mRNAs in the previously capsaicin-injected area 7 days post-capsaicin did not differ either between the ipsilateral and contralateral sides or between the capsaicin control and capsaicin plus 40°C group. Taken together, these data show that persistent mechanical hypersensitivity in the capsaicin plus 40°C group arises despite no clear evidence of ongoing inflammation at the previous injury site, suggesting that this animal model is in the nociplastic pain state. Therefore, henceforth, we called this model a "nociplastic pain" model.

Persistent mechanical hypersensitivity in our nociplastic pain model was alleviated by pain medications

Human subjects who receive intradermal capsaicin injection report increased pain to mechanical stimulation of areas around the injection site (LaMotte, Lundberg, and Torebjörk 1992; LaMotte et al. 1991; Magerl et al. 2001). Capsaicin-induced mechanical hypersensitivity in animals is also regarded as increased mechanical nociception. In fact, this hypersensitivity in animals is effectively alleviated by known pain medications such as morphine and gabapentin (Joshi et al. 2006). Thus, we determined whether the same pain medications would inhibit the prolonged capsaicin-induced mechanical hypersensitivity in our model. As shown in **Fig. 11**, both morphine and gabapentin immediately and robustly inhibited the persistent mechanical hypersensitivity present at days 7-10 post-capsaicin in both males and females. These results also indicated the

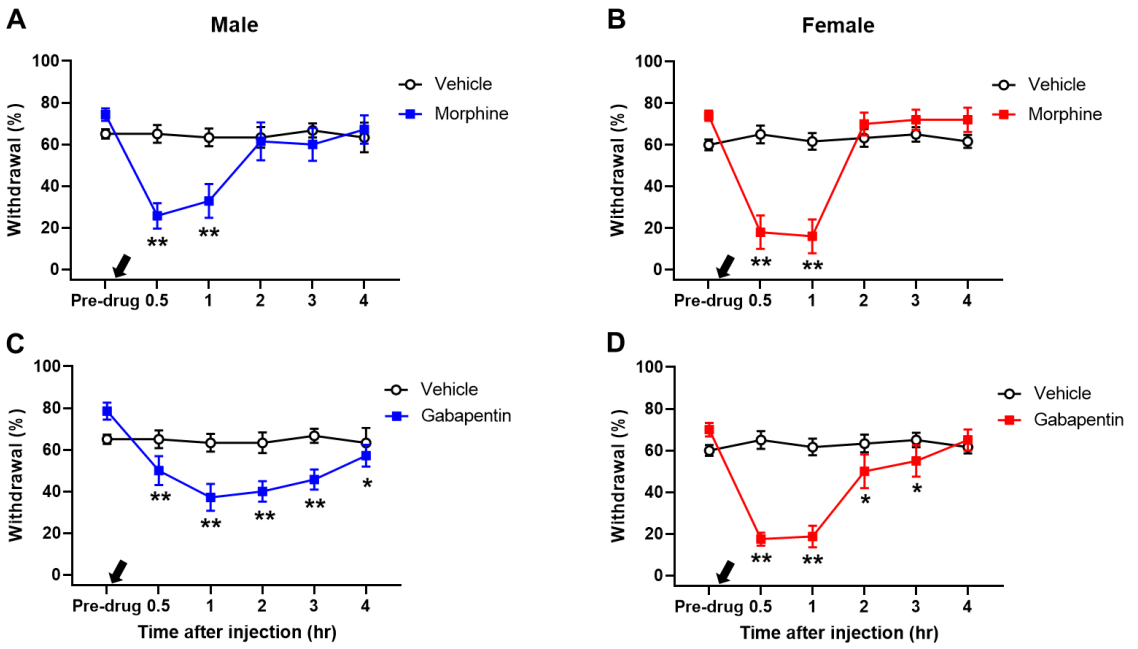

Figure 11: Alleviation of persistent mechanical hypersensitivity by pain medications.

In both males and females, morphine (5 mg/kg, black arrow, A and B) and gabapentin (100 mg/kg, black arrow, C and D) significantly alleviated the persistent mechanical hypersensitivity at 7 to 10 days after stimulating the capsaicin-injected area with 40°C water. Males: n=6 in vehicle (Veh), n=7 in morphine, and n=7 in gabapentin. Females: n=6 in Veh, n=5 in morphine, and n=8 in gabapentin. *p<0.05, **p<0.01 vs pre-drug level in each group by sequential Sidak multiple comparison tests.

nociceptive quality of the observed persistent mechanical hypersensitivity in our nociplastic pain model.

Persistent mechanical hypersensitivity in females was maintained by ongoing afferent activity at the previous injury site

Having determined that our nociplastic pain model manifests persistent mechanical hypersensitivity in the absence of ongoing inflammation in both males and females, we further tested whether this hypersensitivity outside the capsaicin injection area was maintained by ongoing afferent activity at the capsaicin injection (i.e., previous injury)

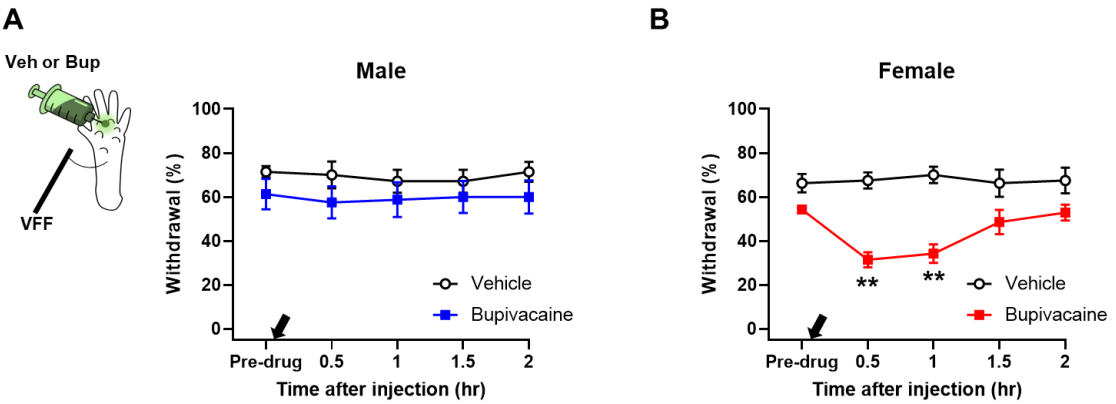

Figure 12: Female-specific alleviation of persistent mechanical hypersensitivity by local anesthesia of previously capsaicin-injected area.

Although a local injection of bupivacaine (Bup, 0.75%, black arrow) at the previous injury (i.e., capsaicin injection) area had no effect on persistent mechanical hypersensitivity outside the previous injury area in males (A), it significantly alleviated the hypersensitivity in females (B) at 7 to 10 days after stimulating the capsaicin-injected area with 40°C water. Males: n=7 in vehicle (Veh) and n=8 in Bup. Females: n=8 in Veh and n=7 in Bup. $^{**}p<0.01$ vs pre-drug level in each group by sequential Sidak multiple comparison tests. VFF, von Frey filament.

area. It was reported that activity of peripheral afferents at the previously injured area in CRPS patients was critical for maintaining chronic mechanical allodynia remote from the injured area (Gracely, Lynch, and Bennett 1992). To determine whether persistent mechanical hypersensitivity was similarly maintained by such afferent activity in our model, we locally injected bupivacaine at the previously capsaicin-injected area 7-10 days post-capsaicin to silence afferents innervating the area. As shown in **Fig. 12**, bupivacaine treatment significantly attenuated persistent mechanical hypersensitivity outside of the treatment area in females but not in males. These data indicate the involvement of sexually dimorphic mechanisms in our nociplastic pain model; specifically, that ongoing activity of afferents innervating the previous injury site plays a

critical role in maintaining the nociceptive system sensitization underlying persistent mechanical hypersensitivity outside the injury area in females but not in males.

Persistent mechanical hypersensitivity in males is maintained by activated microglia in the spinal cord

Recent studies revealed that activated microglia play a male-specific role in other chronic pain models (Mapplebeck et al. 2018; Sorge et al. 2015; Taves et al. 2016). To obtain insights into the maintenance mechanism of the persistent mechanical hypersensitivity in the male nociplastic pain model, we next investigated the role of microglia in the spinal cord. To this end, we determined the effect of Mac-1-saporin, a microglia targeting toxin, on the persistent mechanical hypersensitivity 7-10 days post-capsaicin. We observed that Mac-1-saporin treatment significantly attenuated persistent mechanical hypersensitivity in males, but not in females (**Fig. 13A and B**). We immunostained spinal cord samples from these mice and quantified the immunoreactivity of Iba1, a protein upregulated in activated microglia (Daisuke Ito et al. 1998). As shown in Fig. **13C and D**, in the male nociplastic pain model treated with control saporin, Iba1-immunoreactivity in the ipsilateral dorsal horn was greater than in the paired contralateral side ($t(10)=6.45$, $p<0.001$ by Bonferroni test for 4 pairwise comparisons), but not in the female model. We were additionally able to replicate this experiment in our male capsaicin plus vibration model (**Fig. 14**). Such a difference in Iba1-immunoreactivity between ipsi- and contra-lateral dorsal horns were not detected in the nociplastic pain models treated with Mac-1-saporin, suggesting the effectiveness of Mac-1-saporin treatment on microglial inhibition

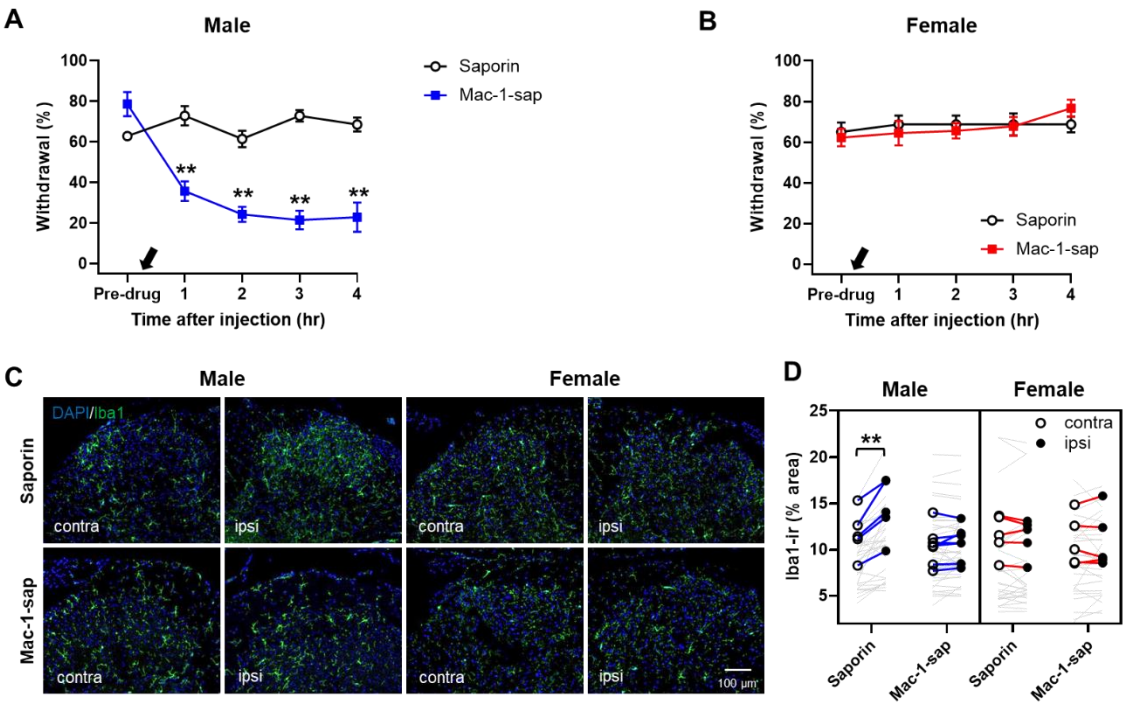

Figure 13: Male-specific alleviation of persistent mechanical hypersensitivity by inhibition of spinal microglia.

An intrathecal injection of the microglia-targeting toxin Mac-1-saporin (Mac-1-sap, 8.85 mM, black arrow) significantly alleviated persistent mechanical hypersensitivity in males (A) but not in females (B) at 7-10 days after stimulating the capsaicin-injected area with 40°C water. $^{**}p<0.01$ vs pre-drug level in each group by sequential Sidak multiple comparison tests. (C and D) The immunoreactivity of Iba1, a marker of activated microglia, was significantly higher in the ipsilateral side in the male nociplastic pain model treated with unconjugated saporin (control sap); this increase in Iba1 immunoreactivity (Iba1-ir) in the male nociplastic pain model was not observed after intrathecal Mac-1-sap treatment. No difference was detected between either dorsal horn sides or treatment groups in the female nociplastic pain model. Males: n=7 in each group. Females: n=8 in control sap and n=9 in Mac-1-sap. $^{**}p<0.01$ by Bonferroni tests for 4 pairwise comparisons in each sex. In D, thick lines with circle symbols indicate the mean Iba1-ir of multiple sections from each mouse. Thin gray lines indicate Iba1-ir in each section.

in our experiments. Of note, the dose of Mac-1-saporin used in this experiment appeared to "normalize" the upregulated Iba1 expression rather than to inhibit the expression below the control level. Together, these data indicate that in males, but not in females, our nociplastic pain model leads to activation of spinal microglia, which maintain the persistent mechanical hypersensitivity.

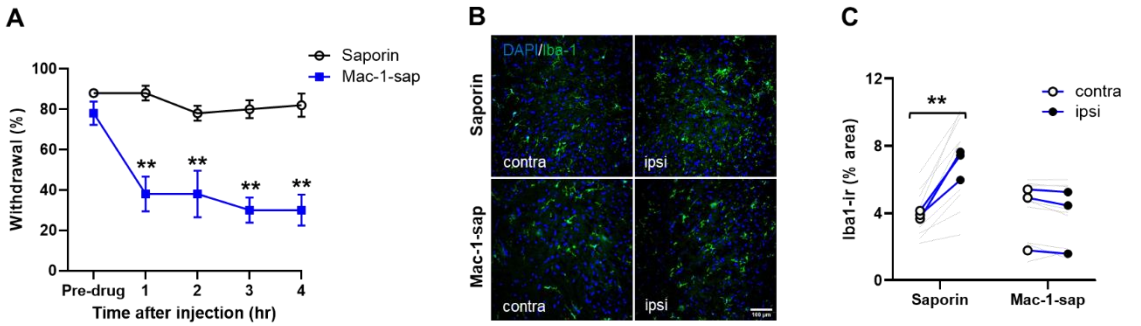

Figure 14: Activated spinal microglia maintain persistent mechanical hypersensitivity in the capsaicin plus vibration model of nociplastic pain.

(A) Intrathecal injection of Mac-1-saporin 7-10 days post capsaicin plus vibration attenuates persistent mechanical hypersensitivity. N=5 in each group. $^{**}p<0.01$ vs. pre-drug level in each group by sequential Sidak multiple comparison tests. (B and C) Compared to that in the contralateral side of the spinal cord dorsal horn, immunoreactivity of Iba1, a marker of activated microglia, was significantly increased in the ipsilateral side (to the capsaicin injected/vibrated hind paw) in the male capsaicin plus vibration nociplastic pain model. This increase was not detected in mice treated with intrathecal Mac-1-saporin. N=3 in each group; $^{**}p<0.01$. In C, thick lines with circle symbols indicate the mean Iba1-ir of multiple sections from each mouse. Thin gray lines indicate Iba1-ir in each section.

DISCUSSION

In the present study, we introduce a novel mouse model to facilitate elucidation of mechanisms for the transition to and maintenance of a nociplastic pain state. In this model, post-injury stimulation (40°C water or vibration) was applied to the injured area created by either intraplantar capsaicin injection or plantar incision to trigger the transition from normally resolving pain to persistent nociplastic pain. Using these experimental paradigms, we significantly prolonged the injury-induced mechanical hypersensitivity, modeling pain chronification and thus providing a platform for understanding its mechanisms. Importantly, we did not detect clear evidence of ongoing tissue damage (inflammation) accounting for this persistent mechanical hypersensitivity.

Hence, the phenotypes of our model reflect the transition to a nociplastic pain state as opposed to a persistent nociceptive pain state due to chronic inflammation.

Similar to the hyperalgesic priming (type I) model that has been used for studying mechanisms of pain chronification (C. A. Parada, Reichling, and Levine 2005), we also used a paradigm of an initial injury (to "prime/sensitize" the nociceptive system) followed by a post-injury stimulus to establish pain chronification. However, our model differs from the hyperalgesic priming model in multiple aspects. First, in our nociplastic pain model, sensitization of the nociceptive system by an acute injury *transitions* to a persistent state (as a continuum) by post-injury stimulation, as the post-injury stimulation must be given before the initial injury-induced hypersensitivity substantially abates to ensure the transition to the nociplastic pain state. By contrast, in the hyperalgesic priming model, chronification is *precipitated* by the post-injury stimulation (most commonly an injection of inflammatory mediators) given after the initial injury-induced hypersensitivity completely resolves. Additionally, while the injury area is probed for detecting mechanical hypersensitivity in the hyperalgesic priming model (i.e., mainly focusing on peripheral nociceptor sensitization), areas outside the initial injury are probed in our nociplastic pain model. This approach makes it possible to infer the involvement of central sensitization in nociplastic pain as central sensitization mediates such "secondary mechanical hypersensitivity". We acknowledge, however, that definitive evidence of a lack of peripheral sensitization in the probed area is necessary to attribute the persistent mechanical hypersensitivity in our model solely to central sensitization. With these similar and dissimilar features to hyperalgesic priming models, our nociplastic pain

model provides novel and complementary systems to investigate the mechanisms of pain chronification.

One of the interesting behavioral phenotypes of this nociplastic pain model (capsaicin plus 40°C group) is that only mechanical, not thermal, hypersensitivity can be made persistent by the post-injury stimulation. This finding suggests that pain hypersensitivity in the nociplastic pain state may not be due to a generalized sensitization of the nociceptive system. Although an original injury can cause such generalized sensitization, as indicated by the observation that capsaicin induces both mechanical and thermal hypersensitivity, chronification of pain hypersensitivity seems to occur rather specifically at nociceptive circuits of a given sensory modality. This notion is consistent with the fact that chronic pain patients can be stratified by their sensory profiles (e.g., mechanical hyperalgesia- vs. thermal hyperalgesia-predominant patient groups) (Baron et al. 2017; Vollert et al. 2017). In this regard, it would be an interesting question whether the nature of post-injury stimulation would be a factor for such a "circuit-specific" chronification of nociceptive system sensitization. In this study, we found both warmth and vibration similarly yielded chronification of capsaicin-induced mechanical hypersensitivity. Therefore, at least in the capsaicin model, it could be that the two different stimulation modalities commonly activate polymodal afferents (sensitized by the initial injury) to persistently sensitize central circuits for mechanical nociception. Alternatively, two different (modality-wise) peripheral afferent pathways may converge on the same central targets (sensitized by the initial injury) that drive persistent sensitization of mechanical nociceptive circuits. Interestingly, the absence of persistent thermal hypersensitivity in our nociplastic pain model corresponds to the absence of local

inflammation in the affected hind paw. If there were persistent inflammation, this model would likely show persistent thermal hypersensitivity as do other inflammatory pain models (Ren and Dubner 1999). It should be noted that in this study we did not measure thermal hypersensitivity in mice treated with a different type of initial injury (i.e., plantar incision) or post-injury stimulation (i.e., vibration). Thus, it remains to be investigated whether these two additional models would also manifest a circuit-specific chronification of nociceptive system sensitization.

Clinically, women are disproportionately affected by nociplastic pain (Melchior et al. 2016). We found that females are more susceptible than males to pain chronification in at least two ways. First, pain chronification can be triggered by relatively lower intensity of post-injury stimulation in females than in males, as 30°C thermal stimulation post-capsaicin prolonged the capsaicin-induced mechanical hypersensitivity only in female mice. Second, compared with males, females have a wider timeframe in which post-injury stimulation can trigger pain chronification. Therefore, if any post-injury events stimulating an injured area occur after a while at low intensity, more females than males are likely to develop a nociplastic pain condition. Our nociplastic pain model is expected to be a valuable tool to further understand the mechanisms underlying these sex differences in the stimulus responsiveness and resolution of sensitized nociceptive system.

In addition to the abovementioned sex differences, we have identified sexually dimorphic mechanisms maintaining the nociplastic pain state. We found that silencing afferents innervating the previous capsaicin injection area significantly attenuated persistent mechanical hypersensitivity outside the injection site only in females. This

"peripherally-maintained" mechanical hypersensitivity observed in our model is reminiscent of the clinical report on 4 CRPS cases (3 women and 1 man) whereby local anesthesia of previous injury sites abolished chronic mechanical allodynia in areas remote from the injury sites (Gracely, Lynch, and Bennett 1992). It remains to be identified how and what afferents are persistently active at the previous injury site to mediate the maintenance of the nociplastic pain state in females. With respect to this, it is noteworthy that 1 day after capsaicin injection, the amounts of IL-6 and TNF-α mRNAs in the injection area were significantly greater in female capsaicin plus post-injury thermal stimulation group than in capsaicin controls. Considering that peripheral injection of IL-6 or TNF-α induces hyperalgesic priming in nociceptors (Dina, Green, and Levine 2008; Hendrich et al. 2013; Carlos A. Parada et al. 2003), it could be that these cytokines (elevated by post-injury thermal stimulation only in females) prime or sensitize nociceptors innervating the capsaicin injection site by mechanisms similar to hyperalgesic priming. As to the identity of such sensitized nociceptors maintaining the nociplastic pain state in female mice, they likely belong to afferent populations not critical for cutaneous heat nociception in the mouse because thermal sensitivity was normal in this nociplastic pain model. Additionally, as female sex hormones, notably estrogen, are implicated as mechanistically important in hyperalgesic priming (Khomula et al. 2017), future studies are warranted to investigate the hormone's potential role in the "peripherally maintained" nociplastic pain state.

Unlike in females, persistent mechanical hypersensitivity in males is "centrally maintained" by activated spinal microglia. This finding corroborates previous studies using chronic neuropathic pain models, in which microglia mediates pain in males

(Echeverry et al. 2017; Kohno et al. 2018), but not in females (Mapplebeck et al. 2018; Sorge et al. 2015). However, those neuropathic pain models and our nociplastic pain model differ in the upregulation of microglial activation markers in females. In chronic neuropathic pain models, microglia in the spinal dorsal horn of female mice are also activated as determined by the upregulation of microglial markers such as Iba1 (Sorge et al. 2015). However, in females, activated microglia appear not to contribute functionally to neuropathic pain per se. In our female nociplastic pain model, by contrast, microglial activation was not detected, suggesting that spinal microglia in female mice may respond differently in neuropathic and nociplastic pain conditions. Conversely, it will be interesting to determine whether detailed cellular properties of spinal microglia in male animals are similar in these two different persistent pain conditions.

Spinal microglia can be activated by factors released by afferent activity (Calvo et al. 2011; Clark and Malcangio 2014; Inoue, Tsuda, and Koizumi 2005; Sawada et al. 1990). For example, a brief intense electrical stimulation of C-fibers, but not Aβ/δ-fibers, led to the activation of microglia in the spinal dorsal horn of male rats, inducing mechanical hypersensitivity which lasted longer than that produced by intraplantar capsaicin injection (Hathway et al. 2009). These observations indicate the possibility that, in our male nociplastic pain model, peripheral sensitization by capsaicin injection allows the sensitized C-fibers to directly activate spinal microglia in response to post-injury stimulation at “normally innocuous” intensity. Alternatively, central sensitization induced by the capsaicin injection may prime male spinal microglia to be readily activated by Aβ/δ-fiber inputs generated by post-injury stimulation.

In conclusion, we have developed a novel mouse model that meets the criteria of IASP's nociplastic pain definition: persistent pain arising from altered nociception despite no clear evidence of actual or threatened tissue damage. This model recapitulates that severe pain after injury and female sex are risk factors for pain chronification, suggesting the significance of intensive pain management after an injury for the prevention of chronic nociplastic pain development. We expect that this model will be a useful tool for providing mechanistic insight to the transition to and maintenance of the nociplastic pain state and will assist in the development of therapeutic interventions for both male and female nociplastic pain syndromes.

Chapter 4: Microglia mediate the transition to and maintenance of nociplastic pain in males

INTRODUCTION

Nociplastic pain encompasses a multitude of chronic pain conditions including complex regional pain syndrome (CRPS) type I, non-neuropathic chronic post-surgical pain, and fibromyalgia (Kosek et al. 2016). These conditions are often incited by an injury which subsequently heals (McCabe and Blake 2008; Steegers et al. 2008), but pain persists despite no detectable tissue or nerve damage, distinguishing this type of pain (i.e., nociplastic pain) from chronic inflammatory and neuropathic pain. It is possible that the transition to nociplastic pain may be triggered during the inciting injury-induced pain hypersensitivity that normally resolves as the injury heals. This notion is supported by the murine model developed in the previous chapter, in which application of normally innocuous stimuli to an injured area triggers a transition to a nociplastic pain state only during the timeframe of substantial pain hypersensitivity after the acute injury (K. Hankerd et al. 2021). When considering that nociceptor inputs from the acute injury (e.g., capsaicin injection) inhibit spinal inhibitory neurons to allow innocuous stimuli to activate nociceptive circuits (Pernía-Andrade et al. 2009), as envisaged in the Gate Control Theory of Pain (Melzack and Wall 1965), it asks the question of whether this spinal disinhibition is a pivotal factor for the transition to a nociplastic pain state.

In the previous chapter, we reported that a nociplastic pain state in our model is maintained by sexually dimorphic mechanisms. Specifically, spinal microglia maintain a nociplastic pain state only in males, as in chronic inflammatory and neuropathic pain models (Mapplebeck et al. 2018; Sorge et al. 2015; Taves et al. 2016). While this

suggests that reactive spinal microglia are a common culprit for *chronic pain* in males, it raises a question of whether spinal microglia activation "drives" the transition to the nociplastic pain state, and if so, whether this activation also utilizes a critical molecular pathway identified in other chronic pain conditions. In addition, considering that nociplastic pain differs from chronic inflammatory or neuropathic pain, it is important to understand how spinal microglia can be activated without chronic tissue injury or direct nerve damage. Therefore, in this study, we investigated whether spinal microglia activation drives the transition to the nociplastic pain state, and if so, how they are activated by normally innocuous stimuli in the context of spinal disinhibition. Portions of these studies have been reported in abstract form (K. McDonough, La, and Chung 2021; K. E. McDonough et al. 2019).

RESULTS

Microglial inhibition prevents the transition to a nociplastic pain state in males

We have previously shown that intraplantar capsaicin injection followed by postinjury stimulation (either warm water immersion or vibration of the capsaicin-injected area) produces mechanical hypersensitivity persisting beyond the normal resolution time in the absence of ongoing peripheral injury (K. Hankerd et al. 2021). Notably, this nociplastic mechanical hypersensitivity was found to be mediated by activated spinal microglia only in males (K. Hankerd et al. 2021). While this result clearly indicates that activated spinal microglia maintain the nociplastic pain state in male mice, it is unknown whether the transition to the nociplastic pain state is driven by spinal microglia activation. To address this question, we inhibited spinal microglia by intrathecally injecting Mac-1-saporin (**Fig.**

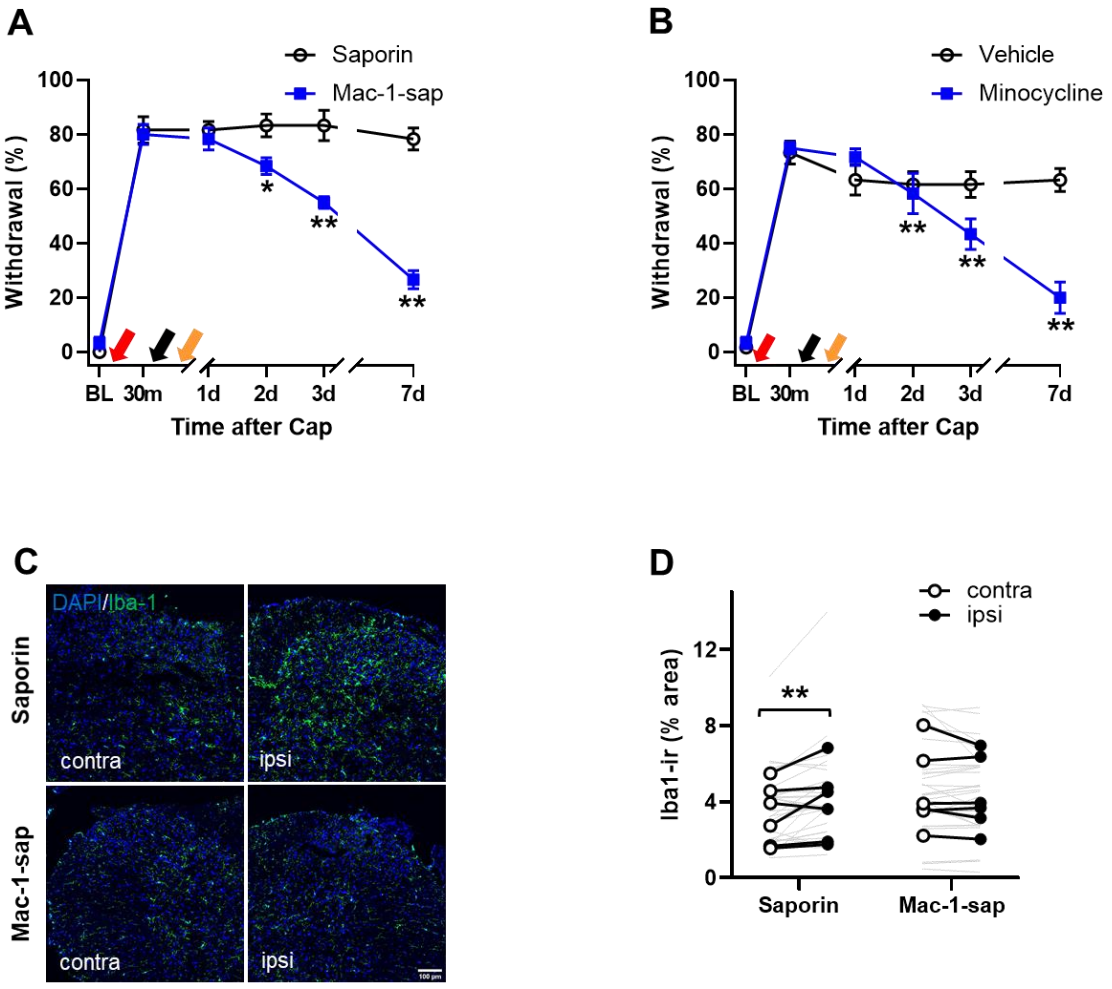

Figure 15: Inhibiting spinal microglia prior to vibration stimulation prevents the transition to a nociplastic pain state.

Intrathecal injection (black arrow) of (A) Mac-1-saporin or (B) minocycline after capsaicin injection (red arrow) but prior to vibration (orange arrow) prevents the transition to persistent mechanical hypersensitivity. N=6 in each group; **p*<0.05, ***p*<0.01 vs. pre-drug level in each group by sequential Sidak multiple comparison tests. (C and D) Compared to the contralateral side of the spinal cord dorsal horn, the ipsilateral (to the capsaicin injected/vibrated hind paw) immunoreactivity of Iba1, a marker of activated microglia, was significantly increased in the male capsaicin plus vibration nociplastic pain model. This increase was not detected in mice treated with intrathecal Mac-1-saporin. N=5 in saporin control group, n=6 in Mac-1-saporin group; ***p*<0.01. In D, thick lines with circle symbols indicate the mean Iba1-ir of multiple sections from each mouse. Thin gray lines indicate Iba1-ir in each section.

15A) or minocycline (**Fig. 15B**) following capsaicin injection, and then applied vibration stimulation 2 hours after the capsaicin injection. When spinal microglia were inhibited at

the time of vibration stimulation, male mice did not develop nociplastic mechanical hypersensitivity (**Fig. 15A and B**: Mac-1-saporin: $F(1,6)=10.29$; $p=0.02$, Minocycline: $F(1,10)=5.423$, $p=0.04$).

We previously found increased immunoreactivity of Iba1, a protein which is upregulated in activated microglia (Daisuke Ito et al. 1998), in the ipsilateral (to the capsaicin-injected hind paw) dorsal horn during the maintenance phase of nociplastic pain state in males (K. Hankerd et al. 2021). In the present experiment, we likewise found that control mice (no microglia inhibition before the postinjury stimulation) displayed the expected increase in Iba1 immunoreactivity in the dorsal horn ipsilateral to the initial intraplantar capsaicin injection compared to the contralateral side. However, mice treated with Mac-1-saporin before the postinjury vibration stimulation did not show a significant difference in Iba1 immunoreactivity between the ipsilateral and contralateral dorsal horns (**Fig. 15C,D**: Saporin: $F(1,62)=14.82$ $p<0.01$ Mac-1-saporin: $F(1,62)=1.81$, $p=0.18$).

The BDNF-TrkB pathway does not mediate the transition to a nociplastic pain state in males

Having found that spinal microglial activation is necessary for postinjury vibration stimulation to produce a nociplastic pain state in males, we next sought to identify the mechanism by which spinal microglia become activated upon the postinjury stimulation. In animal models of neuropathic and inflammatory pain, microglial activation has been shown to depend on the activation of the TrkB receptor by its ligand, Brain-derived

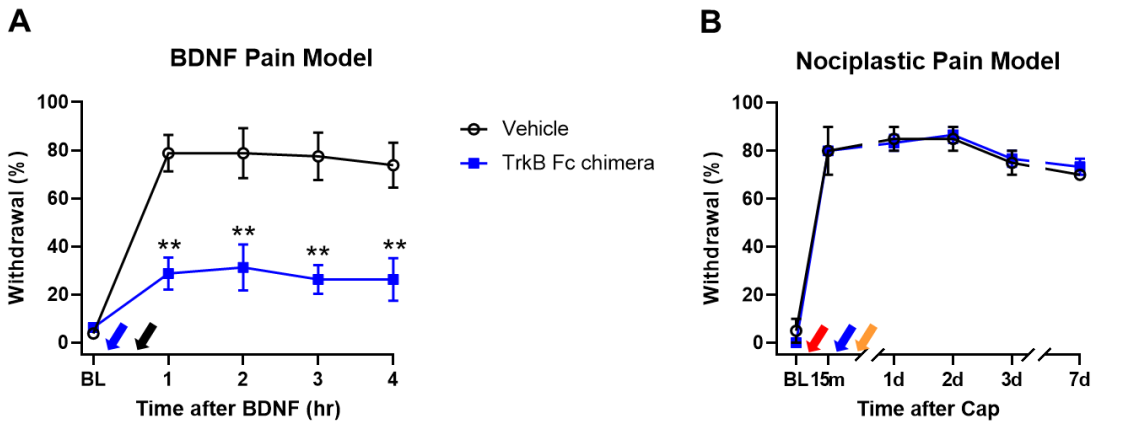

Figure 16: BDNF-TrkB pathway is not responsible for the activation of microglia and the transition to a nociplastic pain state in males.

(A) In order to confirm the effectiveness of our chosen dose and timing of TrkB Fc chimera injection, we tested it in a BDNF pain model. When male mice were pre-treated with intrathecal TrkB Fc chimera (blue arrow), there was a significant attenuation in BDNF (black arrow) induced mechanical hypersensitivity. N=8 in each group. $^{**}p<0.01$ vs vehicle control by sequential Sidak multiple comparison tests. (B) Intrathecal injection of TrkB Fc chimera (blue arrow), which sequesters BDNF, following capsaicin injection (red arrow) but before vibration (orange arrow) failed to prevent the transition to persistent mechanical hypersensitivity. N=5 in each group.

neurotrophic factor (BDNF) (Coull et al. 2005; Ding et al. 2020; Obata and Noguchi 2006; Renn et al. 2011). Therefore, we examined if the activation of TrkB receptors by BDNF would similarly mediate the spinal microglia-driven transition to a nociplastic pain state. To this end, we first validated our experimental approach to inhibit TrkB receptor activation by confirming the efficacy of intrathecal TrkB Fc chimera pretreatment, which sequesters BDNF, against intrathecal BDNF-induced mechanical hypersensitivity (**Fig. 16A**: $F(1,22)=8.10$; $p<0.01$). Having established that TrkB Fc chimera was effective in preventing BDNF-induced mechanical hypersensitivity in our hands, we intrathecally injected TrkB Fc chimera following capsaicin injection but prior to vibration stimulation. This inhibition of the BDNF-TrkB pathway did not prevent the

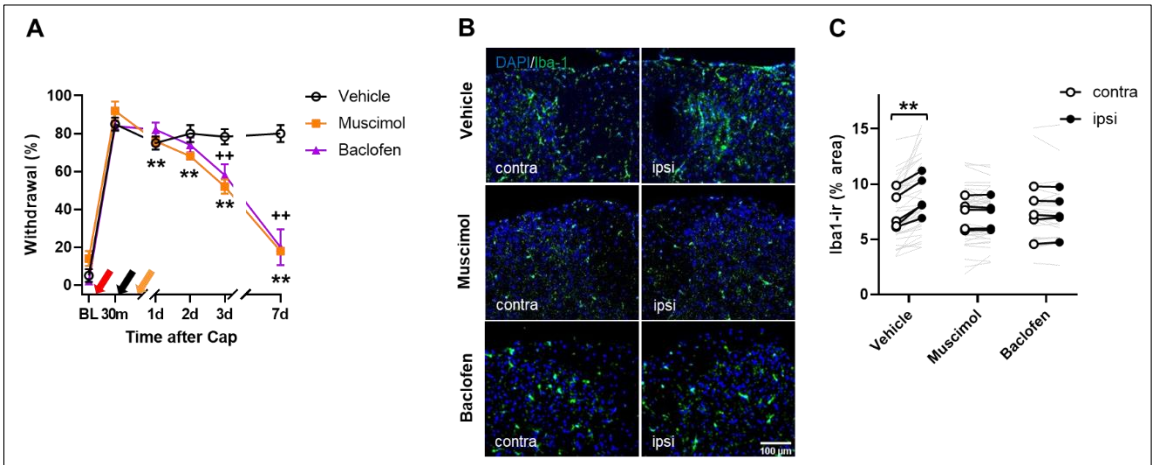

Figure 17: GABA agonist injection prior to vibration stimulation prevents the transition to a nociplastic pain state.

(A) Intrathecal injection (black arrow) of the $GABA_A$ receptor agonist muscimol or the $GABA_B$ receptor agonist baclofen after capsaicin injection (red arrow) but prior to vibration (orange arrow) prevent the transition to persistent mechanical hypersensitivity in males. N=6 in vehicle group, n= 5 in muscimol group, and n=5 in baclofen group; ++p<0.01 vs. pre-drug level in baclofen-treated group, **p<0.01 vs. pre-drug level in muscimol-treated group by sequential Sidak multiple comparisons test. (B and C) Compared to the contralateral side of the spinal cord dorsal horn, the ipsilateral (to the capsaicin injected/vibrated hind paw) immunoreactivity of Iba1, a marker of activated microglia, was significantly increased in the male capsaicin plus vibration nociplastic pain model. This increase was not detected in mice treated with intrathecal muscimol or baclofen. N=5 in each group; **p<0.01. In C, thick lines with circle symbols indicate the mean Iba1-ir of multiple sections from each mouse. Thin gray lines indicate Iba1-ir in each section.

development of nociplastic mechanical hypersensitivity (**Fig. 16B**: F(1,8)=0.01, p=0.92), indicating that the spinal microglia-driven transition to a nociplastic pain state in male mice is not dependent on this neurotrophin signaling pathway.

GABAergic disinhibition underlies the transition a nociplastic pain state in males

The Gate Control Theory of Pain highlights how spinal disinhibition following injury-produced activation of nociceptors allows low-threshold mechanoreceptors (LTMRs) to activate transmission neurons leading to the perception of pain (Melzack 1996; Torsney

and MacDermott 2006). As our vibration stimulation normally activates Aβ-LTMRs, we hypothesized that injury-induced spinal GABAergic disinhibition is critical for postinjury vibration stimulation to trigger the spinal microglia-driven transition to a nociplastic pain state. To test this hypothesis, we increased spinal GABAergic inhibition following capsaicin injection but prior to vibration stimulation by intrathecally injecting muscimol ($GABA_A$ receptor agonist) or baclofen ($GABA_B$ receptor agonist). The GABA agonists prevented the development of nociplastic mechanical hypersensitivity (**Fig. 17A**: Muscimol: $F(4,44)=52.023$, $p<0.01$ Baclofen: $F(4,44)=34.79$, $p<0.01$) and an increase in Iba1 immunoreactivity in the ipsilateral dorsal horn (**Fig. 17B and C**: Vehicle: $F(1,84)=57.31$, $p<0.01$; Muscimol: $F(1,84)=0.00$, $p=0.96$, Baclofen: $F(1,84)=0.01$, $p=0.93$).

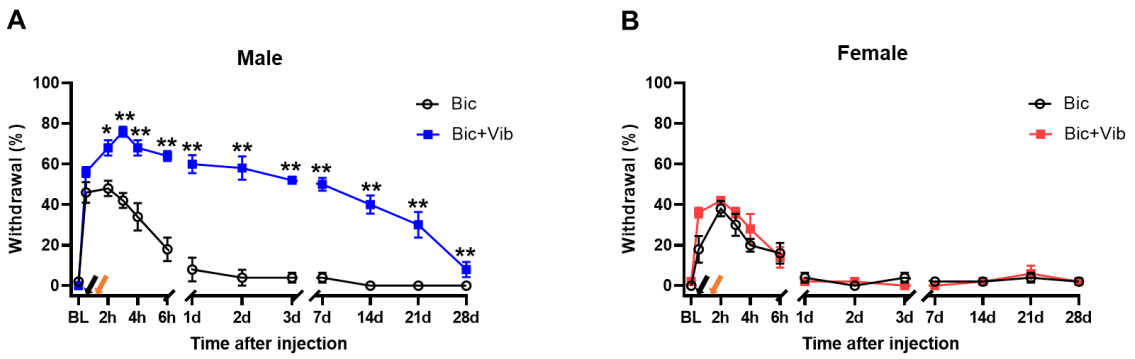

Figure 18: Inhibiting $GABA_A$ receptor signaling prior to vibration stimulation in the absence of an experimental injury can produce a transition to chronic nociplastic pain in males, but not females.

(A) Chronification of bicuculline (black arrow) induced mechanical hypersensitivity by vibration stimulation (orange arrow) occurs in males, lasting at least 21 days, (B) but does not occur in females. Vibration was applied at the time of peak mechanical hypersensitivity response to bicuculline injection: 1h post injection in males, 1.5h post injection in females (Fig. 18). Males: n=5 in each group, females: n=5 in each group. *$p<0.05$, **$p<0.01$ vs bicuculline control by sequential Sidak multiple comparison tests.

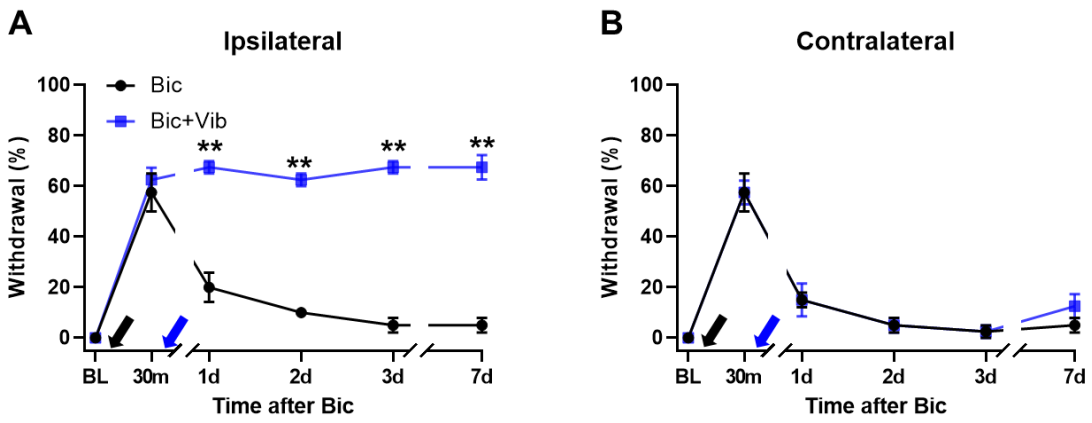

Figure 19: Bicuculline plus vibration produces persistent mechanical hypersensitivity only in the vibrated hind paw.

(A) Intrathecal bicuculline injection (black arrow) plus vibration (blue arrow) produces persistent mechanical hypersensitivity in the vibrated hind paw compared to controls, (B) but mechanical hypersensitivity does not persist in the contralateral hind paw. N=4 per group; **p<0.01.

Conversely, in the next experiment, we investigated if spinal GABAergic disinhibition alone (i.e., without peripheral injury such as intraplantar capsaicin injection) would be sufficient for vibration stimulation to trigger a transition to a nociplastic pain state. Similar to previous reports (I. Lee and Lim 2010; Malan, Mata, and Porreca 2002; Minami et al. 1994), we found that a single intrathecal injection of bicuculline, a $GABA_A$ receptor antagonist, produced transient mechanical hypersensitivity in both male and female mice, which peaks at 1 hour post-injection in males, and 1.5 hours in females (data not shown). Similar results were found for a single injection of the $GABA_B$ receptor antagonist CGP 52432 (data not shown). We applied vibration stimulation at these peak-hypersensitivity timepoints following intrathecal bicuculline injection for each sex. This procedure produced persistent mechanical hypersensitivity lasting at least 21 days in males (**Fig. 18A**: $F(1,12)=96.18$, $p<0.01$) only in the hind paw ipsilateral to the vibration stimulation (**Fig. 19**). In contrast, bicuculline-induced mechanical hypersensitivity was not prolonged by the vibration stimulation in females (**Fig. 18B**: $F(1,12)=0.00$, $p=0.98$),

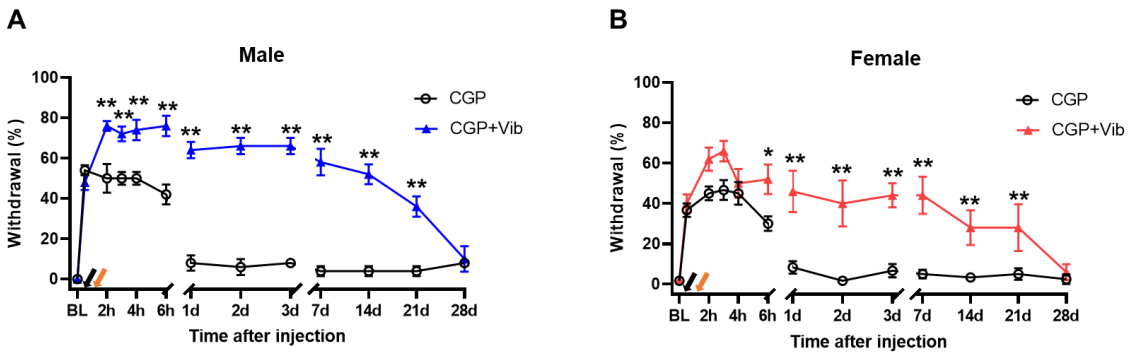

Figure 20: *Inhibiting $GABA_B$ receptor signaling prior to vibration stimulation in the absence of an experimental injury can produce a transition to chronic nociplastic pain in both sexes.*

(A) Chronification of CGP 52432 (black arrow) induced mechanical hypersensitivity by vibration stimulation (orange arrow) occurs in both males and (B) females, lasting at least 21 days. Vibration was applied at 1h post injection in males, 1.5h post injection in females. Males: n=5 in each group, females: n=5 in each group. *p<0.05, **p<0.01 vs CGP 52432 control by sequential Sidak multiple comparison tests.

indicating a dramatic sex-difference in the spinal GABAergic disinhibition-associated mechanisms for producing the transition from acute pain to nociplastic pain.

Likewise, after an intrathecal injection of CGP 52432, unilateral hind paw vibration produced persistent mechanical hypersensitivity in males, lasting at least 21 days (**Fig. 20A**: $F(1,8)=84.39$, $p<0.01$). Interestingly, unlike in female mice that received intrathecal bicuculline, CGP 52432-treated females also developed prolonged mechanical hypersensitivity after vibration stimulation (**Fig. 20B**: $F(1,11)=15.14$, $p<0.01$), indicating that the sex-differences in the spinal disinhibition-associated mechanisms for the transition to a nociplastic pain state are GABA receptor type-specific.

Having determined that a state of nociplastic mechanical hypersensitivity could be produced by spinal disinhibition followed by normally innocuous peripheral stimulation in the absence of an injury in the periphery, we further assessed whether the nociplastic mechanical hypersensitivity produced by bicuculline plus vibration was also

maintained by activated spinal microglia. Thus, 7 days after bicuculline plus vibration, we intrathecally injected Mac-1-saporin. This inhibition of spinal microglia significantly attenuated the persistent mechanical hypersensitivity in male mice (**Fig. 21A**: $F(1,8)=7.10$; $p=0.03$), indicating that spinal microglia contribute to the maintenance of the nociplastic pain state in this bicuculline plus vibration model similar to in the capsaicin plus vibration model. In the spinal cord obtained 7-10 days after bicuculline plus vibration, Iba1 immunoreactivity was found to be increased in the ipsilateral (to the vibrated hind paw) dorsal horn, which not seen following Mac-1-saporin treatment (**Fig. 21B and C**: Saporin ipsi vs. contra: $F(1,58)=26.05$, $p<0.01$; Mac-1-sap ipsi vs. contra: $F(1,58)=0.03$, $p=0.87$).

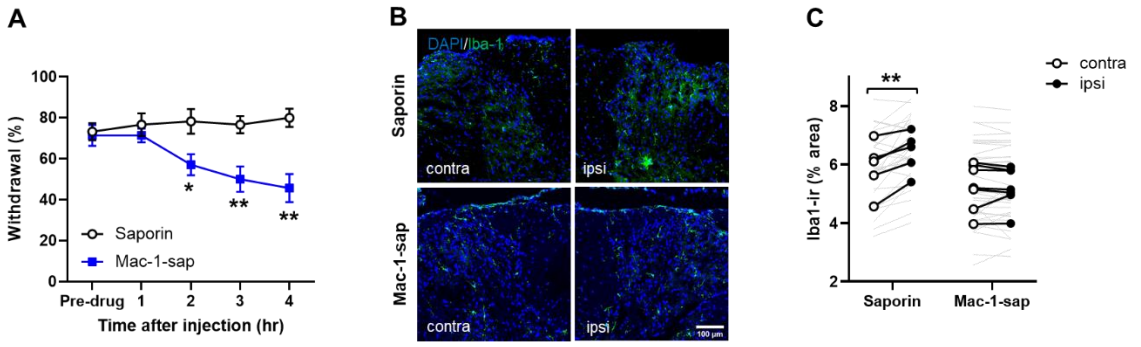

Figure 21: Bicuculline plus vibration model of nociplastic pain is maintained by activated microglia in males.

(A) Intrathecal injection of Mac-1-saporin 7-10 days post bicuculline plus vibration attenuates persistent mechanical hypersensitivity in males. N=7 in Mac-1-saporin treated group, n=6 in saporin treated group. *$p<0.05$ vs. pre-drug level in each group by sequential Sidak multiple comparison tests. (B and C) Compared to the contralateral side of the spinal cord dorsal horn, ipsilateral (to the vibrated hind paw) immunoreactivity of Iba1, a marker of activated microglia, was significantly increased in the male bicuculline plus vibration nociplastic pain model. This increase was not detected in mice treated with intrathecal Mac-1-saporin. N=6 in Mac-1-saporin group, n=5 in saporin group; **$p<0.01$. In C, thick lines with circle symbols indicate the mean Iba1-ir of multiple sections from each mouse. Thin gray lines indicate Iba1-ir in each section.

In the CGP 53432 plus vibration model, the maintenance of the nociplastic pain state was likewise mediated by activated spinal microglia in males (**Fig. 22A**: $F(1,8)=15.26$, $p<0.01$), but not females (**Fig. 22B**: $F(1,8)=0.59$, $p=0.47$). In males treated with CGP 52342 plus vibration, we found a corresponding increase in Iba1 immunoreactivity in the ipsilateral dorsal horn, which was not found in Mac-1-saporin-treated mice (**Fig. 22C and D**: Saporin ipsi vs.contra: $F(1,52)=54.44$, $p<0.01$; Mac-1-sap ipsi vs. contra: $F(1,52)=0.10$, $p=0.76$).

Proinflammatory cytokines, but not prostaglandins, maintain prolonged mechanical hypersensitivity

Activated microglia are known to release a variety of proinflammatory factors which contribute to pain hypersensitivity. Notably, these include proinflammatory cytokines such as IL-6, IL-1β, and TNF-α, as well as prostaglandins generated from the COX pathway, all of which have been implicated in neuropathic pain models (Echeverry et al. 2017; Ma, Du, and Eisenach 2002). Having identified microglia as a key mediator of persistent mechanical hypersensitivity in our male nociplastic pain model, we investigated whether these inflammatory factors played a role in the maintenance of male nociplastic pain. Seven days after capsaicin plus vibration, we intrathecally injected either a cocktail of neutralizing antibodies to inhibit IL-6, IL-1β, and TNF-α, or the COX-inhibitor indomethacin. We observed that mice which had been treated with the cocktail of neutralizing antibodies showed significant attenuation of persistent mechanical hypersensitivity (**Fig. 23A**: $F(1,8)=6.68$, $p=0.03$). However, mice treated with indomethacin showed no change (**Fig. 23B**: $F(1,9)=0.88$; $p=0.37$). These data indicated

that proinflammatory cytokines, but not prostaglandins, play a key role in maintaining prolonged mechanical hypersensitivity in our male model of nociplastic pain.

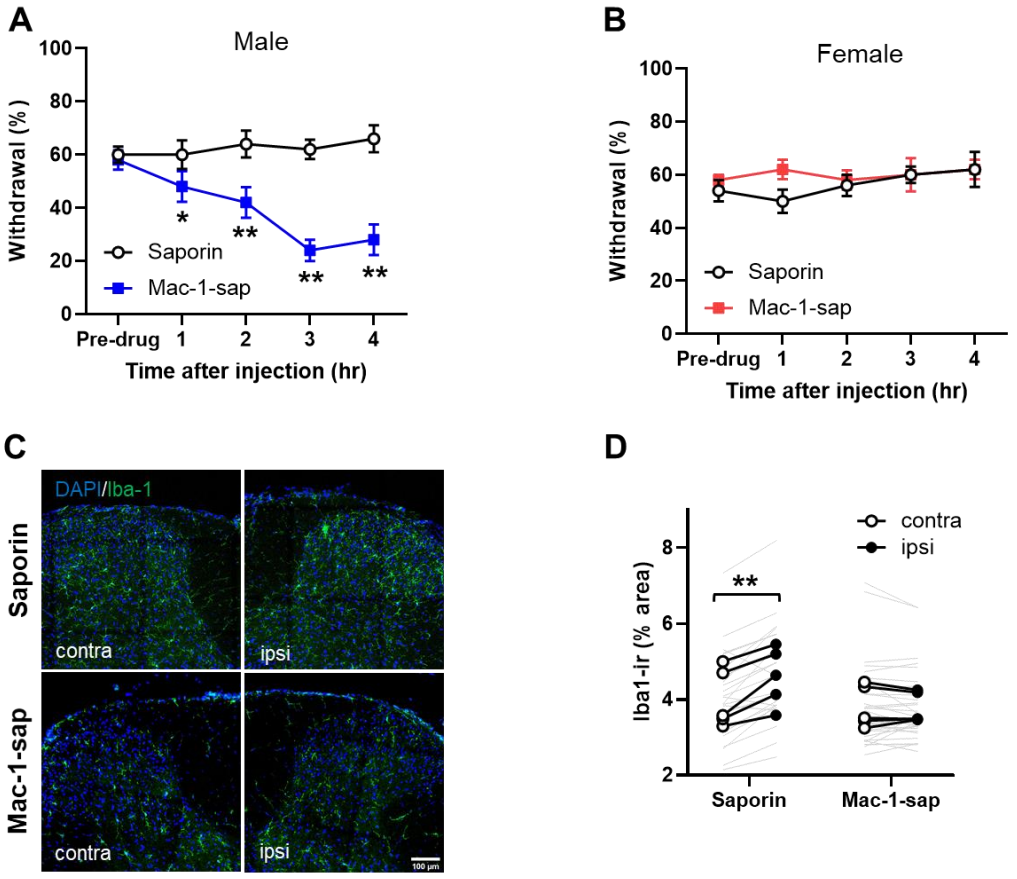

Figure 22: CGP 52432 plus vibration model of nociplastic pain is maintained by activated microglia in males.

(A) Intrathecal injection of Mac-1-saporin 7-10 days post CGP 52432 plus vibration attenuates persistent mechanical hypersensitivity in males, (B) but not females. Males: n=5 in each group, females: n=5 in each group. *p<0.05, **p<0.01 vs. pre-drug level in each group by sequential Sidak multiple comparison tests. (C and D) Compared to the contralateral side of the spinal cord dorsal horn, ipsilateral (to the vibrated hind paw) immunoreactivity of Iba1, a marker of activated microglia, was significantly increased in the male CGP 52432 plus vibration nociplastic pain model. This increase was not detected in mice treated with intrathecal Mac-1-saporin. N=5 in each group; **p<0.01. In D, thick lines with circle symbols indicate the mean Iba1-ir of multiple sections from each mouse. Thin gray lines indicate Iba1-ir in each section.

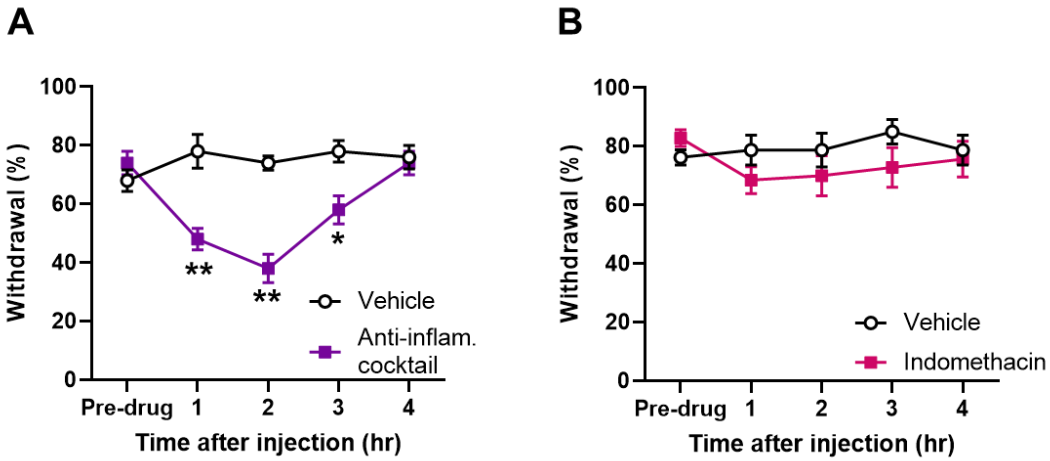

Figure 23: Inhibiting inflammatory cytokines, but not the COX pathway, attenuates the nociplastic pain state in males.

(A) Intrathecal injection of a cocktail of antibodies targeting TNF-α, IL-1β, and IL-6 delivered 7-10 days post capsaicin plus vibration significantly attenuates persistent mechanical hypersensitivity. N=5 in each group. (B) However, intrathecal injection of indomethacin, targeting the COX pathway, does not alter pain hypersensitivity. N=8 in vehicle group, n=7 in indomethacin group. **p*<0.05, ***p*<0.01 vs. pre-drug level in each group by sequential Sidak multiple comparison tests.

DISCUSSION

In this study we demonstrated that activation of spinal microglia is necessary for the transition from acute injury-induced pain to chronic nociplastic pain in males. However, unlike other forms of chronic pain, the BDNF-TrkB pathway does not mediate this microglial activation. It has been demonstrated that in both inflammatory and neuropathic pain models, BDNF and TrkB receptors are upregulated in the dorsal root ganglion leading to activation of microglia and alterations in pain sensitivity (Ding et al. 2020; Y.-T. Lin et al. 2011; Obata and Noguchi 2006; Zhou et al. 2011). Additionally, endogenous BDNF has been shown to play a key role in producing hyperalgesic priming in males (Moy et al. 2018), and inhibition of BDNF-TrkB interaction has been shown to alleviate hypersensitivity (Coull et al. 2005; Ding et al. 2020; Inoue 2017). In line with these

findings, we showed that inhibiting BDNF-TrkB interactions attenuated hypersensitivity in a purely spinal BDNF-induced pain model (**Fig. 16A**). However, inhibiting BDNF-TrkB interactions could not prevent the transition to persistent mechanical hypersensitivity in our male nociplastic pain model (**Fig. 16B**), highlighting that the mechanisms triggering a transition to nociplastic pain are distinct from those underlying the development of other forms of chronic pain in males despite reactive spinal microglia being a common culprit. It further emphasizes the necessity of finding mechanism-specific targets for the treatment of different types of chronic pain. In this regard, it remains to be investigated what signaling pathways mediate microglia activation for the transition to and maintenance of nociplastic pain in males.

The literature reports that suppression of spinal GABAergic inhibition (i.e. disinhibition) allows Aβ-fibers to activate dorsal horn nociceptive circuitry, resulting in Aβ-fiber-mediated mechanical allodynia (Duan et al. 2014; Melzack and Wall 1965; Peirs et al. 2015). However, whether these Aβ-fibers are also capable of activating microglia within the context of disinhibition remains unknown. Interestingly, it has been shown that in male rats, a brief intense electrical stimulation of C-fibers, but not Aβ- or Aδ-fibers, activates dorsal horn microglia, and induces long-lasting hypersensitivity (Hathway et al. 2009). However, in our model, even vibration, which normally activates Aβ-fibers, produces a transition to long-lasting hypersensitivity, with a key difference being that the pain circuit is already in a sensitized state by C-fiber inputs due to the inciting capsaicin injury (in the capsaicin plus vibration model). Considering that intense nociceptor inputs cause disinhibition in the dorsal horn to result in the activation of nociceptive circuitry by normally innocuous stimuli (Pernía-Andrade et al. 2009), it is

possible that acute injury-induced disinhibition in the dorsal horn plays a key role in the spinal microglial activation by low-threshold afferent inputs and consequently, in the transition from an acute to a nociplastic pain state in males. Here, we provided support for this hypothesis by showing that boosting spinal GABAergic inhibition at the time of postinjury vibration stimulation prevented the prolongation of mechanical hypersensitivity and the activation of spinal microglia (**Fig. 17**). We further demonstrated that, even in the absence of an acute peripheral injury, inhibiting either $GABA_A$ or $GABA_B$ receptors at the spinal level and applying vibration stimulation was sufficient for producing a nociplastic pain state in males (**Fig. 18A, 20A**), which is similarly mediated by activated microglia (**Fig. 21, 22A, C and D**). Collectively, these findings suggest that spinal microglia can be activated even by low-threshold afferent inputs in males; however, spinal GABAergic inhibition is keeping such microglia activation in check.

Interestingly, in females, we saw that inhibiting $GABA_B$ receptors (with CGP 52342), but not $GABA_A$ receptors (with bicuculline), prior to vibration was sufficient to produce a nociplastic pain state. While the cause of this receptor-specific sex-difference requires further investigation, this study clearly demonstrates that in the female CGP 52432 plus vibration model, the nociplastic pain state is not mediated by spinal microglia unlike in its male counterpart (**Fig. 22**). The male-specific spinal microglia activation by LTMR inputs in the spinal GABAergic disinhibition state begs a question of whether spinal microglia are under a different type of inhibitory control in females. It is our immediate interest in future study to examine whether spinal *glycinergic* disinhibition is different from the *GABAergic* one in terms of sex-dependent spinal microglia activation by LTMR inputs.

As mentioned above, it remains to be elucidated by what mechanisms the nociplastic pain is induced and maintained in female CGP 52432 plus vibration model. In our previous study, ongoing primary afferent activity at the previously capsaicin-injected area was found to maintain the nociplastic pain state in the female capsaicin plus warmth model (K. Hankerd et al. 2021). Considering that $GABA_B$ receptor activity modulates the release of glutamate from C-fiber central terminals (Ataka et al. 2000; Wang et al. 2007), it could be that inhibition of $GABA_B$ receptors potentially allow ongoing activity of central terminals of primary afferents in females, driving the nociplastic pain state.

Activated microglia are known to cause neuroinflammation in the central nervous system. In line with this, we found that the nociplastic pain state in males, shown to be microglia-driven in both our present and previous studies, was maintained by proinflammatory cytokines TNF-α ,IL-1β, and IL-6 (**Fig. 23A**), all of which are known to be released from microglia and have been shown to play a key role in nociception, including chronic neuropathic pain (Echeverry et al. 2017; Ji, Xu, and Gao 2014). Interestingly, prostaglandins also play a key role in other pain conditions and are also released by microglia (Kanda et al. 2013; Ma, Du, and Eisenach 2002), but were not found to play a significant role in our nociplastic pain model. This may be a result of differences in how spinal microglia are initially activated in different pain models, and further research is warranted.

In conclusion, we have shown that both the transition to and maintenance of male nociplastic pain is mediated by the complex interplay between the spinal GABAergic inhibitory system and spinal microglia (**Fig. 24**). In males, disinhibition at the level of the spinal cord is both sufficient and necessary to produce a transition to a nociplastic pain

state when followed by postinjury activation of low-threshold afferents (e.g., by vibration stimulation). These findings indicate that preemptively inhibiting spinal microglia or increasing spinal inhibitory tone during acute nociceptive pain may help prevent the development of nociplastic pain in males.

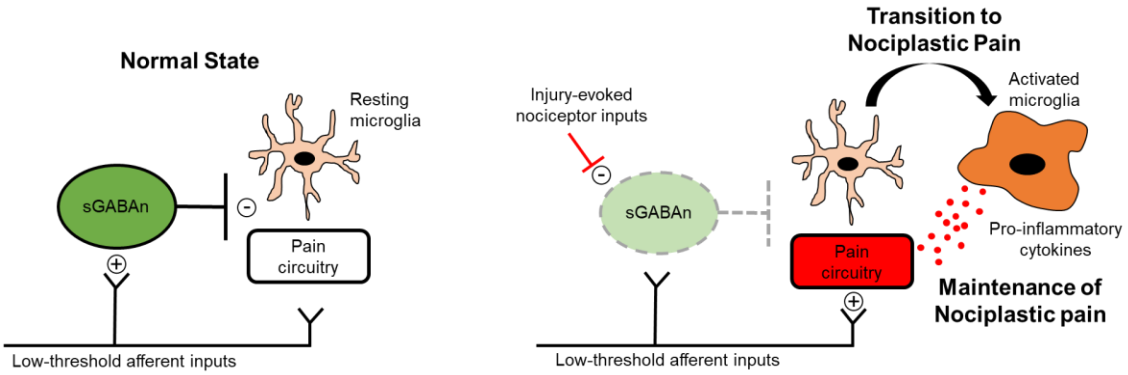

Figure 24: Proposed mechanism for the transition to and maintenance of a nociplastic pain state in males.

Under normal conditions, microglia remain in a non-activated "resting" state and sGABAn prevent low-threshold afferent inputs from activating pain circuitry. However, in males, when injury-evoked nociceptor inputs inhibit sGABAn, low-threshold afferent inputs are not only able to activate pain circuitry (as proposed by the Gate Control Theory of Pain), but also activate microglia, producing a transition to a nociplastic pain state which is maintained by pro-inflammatory cytokines.

Figure created by Kathleen McDonough and generated using Microsoft PowerPoint.

Chapter 5: Conclusions and Future Directions

Up until now, there have been no murine models for the specific study of the transition to nociplastic pain. This has prevented in-depth studies of the mechanisms underlying nociplastic pain which may facilitate the future development of effective therapeutics for those who are at the greater risk of developing nociplastic pain conditions after an injury and/or already suffering from these conditions. In this study we aimed to fill this gap by developing a novel model of nociplastic pain (K. Hankerd et al. 2021), and then further investigated the sex-specific mechanisms underlying the transition to and maintenance of the nociplastic pain state.

Here we have shown that normally innocuous peripheral stimulation applied during a period of pain hypersensitivity can produce a nociplastic pain state in both male and female mice, which was maintained by peripheral afferent activity in females[2], and reactive microglia in males. The transition to a nociplastic pain state was not only limited to experimental conditions in which peripheral stimulation was applied to a previously injured hind paw (by either capsaicin injection or incision), but also in the case where mechanical hypersensitivity was initially induced by inhibition of $GABA_B$ receptors in the spinal cord. Furthermore, males, but not females, transitioned to a nociplastic pain state when normally innocuous mechanical stimulation was applied to the periphery (hind paw vibration) following inhibition of $GABA_A$ receptors in the spinal cord. This spinal GABA receptor type-specific sex difference is of great interest to us for further study, and is particularly interesting in light of sex differences related to $GABA_A$ receptor

[2] For those interested in female-specific mechanisms, I recommend reading Dr. Kali Hankerd's dissertation, "Female-Specific Mechanisms of Nociplastic Pain in Murine Model" (K. Hankerd 2021).

functioning in the periaqueductal gray, which have been identified in inflammatory pain (Tonsfeldt et al. 2016). It is also interesting to note that the development of a nociplastic pain state in females due to inhibition of $GABA_B$ receptors followed by vibration points toward an additional female mechanism differing from what we found in Chapter 3. As previously mentioned, in our initial capsaicin plus postinjury stimulation model of nociplastic pain, female nociplastic pain was dependent upon afferent activity at the initial capsaicin injury site. However, there is no such inciting peripheral injury in our CGP 52432 plus vibration model. Further research into female nociplastic pain in the absence of ongoing peripheral afferent activity would be an interesting avenue of further research. Furthermore, forming a greater understanding of how differences in GABAergic functioning underlie the sex-specific central sensitization, as well as how this is impacted by region and pain type may help us to better understand how male- or female-specific mechanisms of pain are utilized and potentially reversed.

Having shown in the first half of this study that male and female nociplastic pain was maintained by sex-specific mechanisms, we sought to further analyze the mechanisms underlying the male nociplastic pain state in the second half. In brief, we found that in males, not only are activated spinal microglia maintaining the nociplastic pain state, but they are also required for the transition from acute pain to nociplastic pain. However, if spinal GABAergic signaling is increased at the time of transition, microglia do not become activated by postinjury stimulation, preventing the development of the nociplastic pain state. Finally, during the maintenance phase of the nociplastic pain state, proinflammatory cytokines known to be released from microglia mediate persistent mechanical hypersensitivity.

We additionally found that microglia activation occurs when peripheral stimulation able to activate LTMR is applied while spinal GABAergic disinhibition, producing acute mechanical hypersensitivity, is in place. This provides evidence for the possibility that, in a state of spinal GABAergic disinhibition, spinal microglia may be directly activated by LTMR activation, similar to evidence that spinal microglia can be activated by high threshold afferent inputs (Hathway et al. 2009). In the future, we are interested in further investigating the molecular mechanism by which microglia may be activated by primary afferents to induce a transition to a nociplastic pain state. It is noteworthy that activation of microglia in our nociplastic pain model was not dependent on the BDNF-TrkB pathway, signifying a clear difference in spinal microglia-mediated mechanisms between nociplastic pain and chronic inflammatory and neuropathic pain (Ding et al. 2020; Y.-T. Lin et al. 2011; Obata and Noguchi 2006; Zhou et al. 2011).

Overall, we have shown that normally innocuous stimuli such as vibration or warmth, when applied following a peripheral injury, produces a transition to a nociplastic pain state in both males and females. The nociplastic pain state is maintained by sex-specific mechanisms: reactive microglia in males, and persistent peripheral afferent activity in females. Furthermore, peripheral vibration stimulation following $GABA_B$ receptor inhibition also produces a transition to a nociplastic pain state in both males and females. We further found that there is a complex interplay between GABAergic signaling and microglia activation within the spinal cord in the male nociplastic pain state. Specifically, spinal microglia become activated upon transition-inducing peripheral stimulation during (acute injury-induced) spinal GABAergic disinhibition, and then activated spinal microglia maintain the disinhibition state, to which proinflammatory

cytokines released from microglia likely contribute. This information provides greater insight into potential targets for sex-specific treatments of nociplastic pain, as well as introducing a potential role for spinal microglia within the Gate Control Theory of Pain, pending further research.

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Vita

Kathleen Erin McDonough was born in San Antonio, Texas on December 19, 1993 to Lupita and William Winters. She received her Bachelor of Science from Baylor University in May of 2016, where she also completed her Honors Thesis in the laboratory of Dr. Bradley Keele. During the summers of 2014 and 2015, she was a Summer Undergraduate Research Fellow in the laboratory of Dr. David Morilak at UT Health Science Center at San Antonio. In 2016, Kathleen joined the Neuroscience Graduate Program at the University of Texas Medical Branch (UTMB) as a Presidential Scholar to pursue a Ph.D. under the mentorship of Dr. Jun-Ho La and Dr. Jin Mo Chung. During her time at UTMB, Kathleen served as a teaching assistant for multiple classes within both the graduate school and medical school. She has also received numerous awards, including the Dr. and Mrs. Seymour Fisher Academic Excellence Award in Neuroscience, and received funding through the NIH Blueprint Diversity Specialized Predoctoral to Postdoctoral Advancement in Neuroscience Award. She and her husband, Dr. William McDonough, reside in Spring, Texas.

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