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FEMALE-SPECIFIC MECHANISMS OF NOCIPLASTIC PAIN IN MURINE MODEL

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FEMALE-SPECIFIC MECHANISMS OF NOCIPLASTIC PAIN IN MURINE MODEL

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Normally resolving pain can transition into chronic nociplastic pain, which predominately affects women. To facilitate mechanistic studies on nociplastic pain, we developed a murine model in which postinjury thermal stimulation of injured area triggers the transition to a nociplastic pain state more readily in females. This model manifested mechanical hypersensitivity outside the previously injured area beyond the normal resolution time without persistent local inflammation. Ongoing afferent activity at the previously injured area maintained the nociplastic pain state only in females. The development of this peripherally maintained nociplastic pain state was female gonadal hormone-dependent, in which estrogen acting through G protein-coupled estrogen receptor was a critical mechanistic player. In gonad-intact females, allyl isothiocyanate (AITC)-sensitive afferents at the previously injured area maintained the nociplastic pain state; these afferents spontaneously at a higher frequency and had a decreased mechanical threshold compared to control or acute pain-resolved state, which was not observed in ovariectomized females and males. These results demonstrate that postinjury stimulation of an injured area triggers the transition from transient pain to nociplastic pain, females are more susceptible to this

transition, and AITC-sensitive afferents at the previously injured area maintain the nociplastic pain state in a female gonadal hormone-dependent manner.

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List of Abbreviations

AITC	Allyl isothiocyanate
CGRP	Calcitonin gene-related peptide
CRPS	Complex regional pain syndrome
GPER	G protein-coupled estrogen receptor 1
IASP	International Association for the Study of Pain
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
OVX	Ovariectomized
OVX+E2	Ovariectomized, estrogen reconstituted
OVX+delayed E2	Ovariectomized, estrogen reconstituted 4 days post-capsaicin
PKCε	Protein kinase C epsilon
QX-314	n-ethyl bromide
TNF-α	Tumor necrosis factor alpha
TRPA1	Transient receptor potential A1
TRPV1	Transient receptor potential V1
$TRPV1^+$	Transient receptor potential V1-expressing

Chapter 1: Introduction

BACKGROUND

Nociceptive circuitry and pain perception

Pain is an aversive response to noxious stimuli (Raja et al., 2020) that facilitates wound healing and survival (Crook et al., 2014). Pain perception is a complex phenomenon resulting from nociceptor activation, activation of sensory-affective regions in the brain, and descending pain modulation. Though nociception, or the activation of nociceptive pathways, does not necessitate pain, pain typically requires the activation of nociceptors that are high-threshold peripheral afferents capable of transducing and encoding noxious stimuli (IASP Task Force, 2012).

Nociceptors are functionally heterogeneous and can be classified into subpopulations based on their conduction velocity, capacity to release afferent-sensitizing peptides, (Dubin & Patapoutian, 2010), response to specific stimulus modalities, and receptor expression profiles. Unmyelinated C-fibers, which are gathered into Remark bundles (Corfas et al., 2004), have a slower conduction velocity than thinly myelinated Aδ-fibers (Cain et al., 2001). Humans perceive this difference in conduction velocity as two temporally distinct waves of pain resulting from simultaneous activation of Aδ-fibers and C-fibers. As these fibers conduct information asynchronously, the initial sharp, well-localized pain is conveyed by Aδ fibers and the second wave of diffuse pain by C-fibers (Price & Dubner, 1977). Nociceptors can be subdivided into peptidergic neurons (which secrete neuropeptides such as substance P (Liu et al., 1997) and calcitonin gene-related peptide (CGRP) (Messlinger et al., 1995)) and non-peptidergic populations. Beyond

conduction velocity and ability to release neuropeptides, nociceptor subpopulations can be delineated by their response to specific stimulus modalities and intensities. For instance, Transient Receptor Potential (TRP) V1 and TRPA1 confer sensitivity to noxious heat (>42°C) (Caterina et al., 1997) and noxious mechanical stimulation (Kwan et al., 2009), respectively. Some of these receptors are additionally activated by pharmacological and/or inflammatory agents. For instance, TRPV1 is activated by capsaicin (Caterina et al., 1997) and TRPA1 by allyl isothiocyanate (AITC) (Takaya et al., 2015). These receptor expression profiles are tissue-specific, as ~61% of afferents innervating the colon co-express TRPV1 and TRPA1, as compared to ~9% of cutaneous afferents, in mice (Malin et al., 2011).

Following activation by noxious stimuli and/or inflammatory and chemical agents, nociceptors relay nociceptive information to the spinal cord dorsal horn. The spinal cord is delineated into laminae, which correspond to specific structural and functional areas in which nociceptive, non-nociceptive, and proprioceptive information is consolidated (Rexed, 1952). Within the superficial lamina (I-II), nociceptor processes synapse upon select populations of second order neurons (Almeida et al., 2004). These second order neurons are critical for nociceptive transmission, as they receive and integrate information from peripheral afferents and adjacent spinal inhibitory and excitatory neurons. In fact, robust activation of inhibitory interneurons by non-nociceptive afferents can block further transmission of nociceptive information (Lu & Perl, 2003; Mendell, 2014).

Second order neurons transmit nociceptive information to the brain via several ascending spinal tracts. Classically, the spinothalamic tract—which decussates before ascending to and terminating in the lateral thalamic nuclei (Hodge & Apkarian, 1990)—

has been studied in pain perception. Spinothalamic tract projections from the lateral thalamic nuclei to the somatosensory cortex convey discriminatory sensory information (Almeida et al., 2004), while projections to the anterior cingulate cortex regulate the affective component of pain (Meda et al., 2019) and affect-regulation (Stevens et al., 2011). In addition to the spinothalamic tract, recent studies have highlighted the importance of the ascending spinoparabrachial tract in the affective-motivational component of pain and aversive responses to noxious stimuli (Chiang et al., 2020; Choi et al., 2020; Raver et al., 2020). Spinoparabrachial tract projections to the central nucleus of the amygdala have been suggested to mediate aversive pain memory formation (Chiang et al., 2020) and chronic pain (Raver et al., 2020; Sun et al., 2020), while collaterals to the lateral periaqueductal gray have been implicated in escape behaviors (Chiang et al., 2020). The parabrachial nucleus is also tied to the mesolimbic dopamine system, which regulates reward-reinforcement (Creed et al., 2014), via collaterals to the ventral tegmental area (Yang et al., 2021). Projections from the parabrachial nucleus to the ventral tegmental area have been implicated in affective disorders, such as depression (L. Zhang et al., 2021), which may be comorbid with chronic pain.

Several of the aforementioned brain regions additionally modulate pain perception via descending projections to the spinal cord. Notably, the rostroventromedial medulla acts as a relay station for descending pain modulation, as it receives projections from the periaqueductal gray (Morgan et al., 2008) and the locus coeruleus (Pertovaara, 2006). In addition to descending modulation, the periaqueductal gray and the locus coeruleus produce antinociception through the release of endogenous opioids (Sims-Williams et al., 2017) and norepinephrine (Pertovaara, 2006).

Peripheral sensitization

In addition to the canonical symptoms of redness (or flare), edema, and heat (Punchard et al., 2004), inflammation causes pain. As previously mentioned, nociceptors may be activated by inflammatory agents, which are secreted by immune cells and peptidergic neurons at the injury area, such as interleukin 6 (IL-6) (Dina et al., 2008), tumor necrosis factor alpha (TNF-α) (Parada et al., 2003), and CGRP (Kilo et al., 1997). This pain is a direct byproduct of nociceptor sensitizing and/or activating agents; thus, peripheral sensitization is usually coupled to peripheral inflammation and is typically restricted to inflamed or injured areas (Hucho & Levine, 2007). Sensitized nociceptors innervating the injured and/or inflamed area may fire spontaneously, have a decreased threshold, and/or an augmented response to suprathreshold stimuli (IASP Task Force, 2012), producing behaviors such as hypersensitivity to mechanical and thermal stimuli. While uncommon, it should be noted that sensitized nociceptors can be rendered persistently active after local inflammation has resolved. This is a result of transcriptional-translational changes that induce long-term changes in nociceptor properties (Aley et al., 2000; Coderre et al., 2004).

Central sensitization and secondary mechanical hypersensitivity

Intense nociceptor inputs can lead to sensitization of spinal and supraspinal nociceptive circuitry, termed 'central sensitization'. Central sensitization can be uncoupled from peripheral input, such that central nociceptive pathways remain sensitized despite the absence of peripheral inflammation or injury (Latremoliere & Woolf, 2009). In addition to synaptic facilitation (IASP Task Force, 2012) and disinhibition, central sensitization is also accompanied by unmasking of injury type-specific circuitry through which Aβ fibers, generally mediating innocuous mechanical sensation, gain access to nociceptive second

order neurons (Peirs et al., 2021). Notably, there is an emergence of dysfunctional behavioral phenotypes, such as secondary mechanical hypersensitivity (Ziegler et al., 1999). Secondary mechanical hypersensitivity is characterized by paradoxical hypersensitivity to normally innocuous and noxious mechanical stimulation of uninjured tissue remote from the original injury (Baumann et al., 1991; La et al., 2017; LaMotte et al., 1991; Torebjörk et al., 1992; Ziegler et al., 1999). This hypersensitivity can be explained, at least in part, by the Gate Control Theory of Pain (Ronald Melzack & Patrick D. Wall, 1965). At the level of spinal circuitry, nociceptive neurons innervating the injured area and afferents (both nociceptors and non-nociceptive Aβ fibers) innervating outside the injured area converge upon inhibitory interneurons within the substantia gelatinosa (Sheikh & Dua, 2020). Aβ fibers, which have a fast conduction velocity, normally block the transmission of pain by nociceptors, through excitation of inhibitory interneurons (Ronald Melzack & Patrick D. Wall, 1965). However, when central sensitization involving disinhibition is present, Aβ fibers cannot effectively conduct such inhibitory modulation, resulting in their access to the nociceptive circuitry and facilitation of pain transmission by nociceptors (Mendell, 2014).

Acute injury-induced pain models

Preclinical and clinical studies on normally resolving pain commonly use moderate doses of capsaicin (0.1%) as an experimental injury. Intradermal capsaicin activates TRPV1-expressing ($TRPV1^+$) nociceptors in the skin (Caterina et al., 1997), eliciting acute pain by direct nociceptor activation. Additionally, as a subset of $TRPV1^+$ nociceptors are peptidergic, capsaicin triggers neurogenic inflammation (Lin et al., 1999). Neurogenic (or sterile) inflammation is characterized by redness, edema, and pain (Chalovich & Eisenberg,

2005). However, unlike pathogen- or injury-induced inflammation, neurogenic inflammation is precipitated the release of a barrage of nociceptor sensitizing agents by peptidergic afferents (Lin et al., 1999). These peptides trigger vasodilation, plasma extravasation, and nociceptor sensitization at the capsaicin-injection area (Frias & Merighi, 2016). At a behavioral level, intradermal capsaicin first induces non-evoked nocifensive behaviors in rodents (Sawynok et al., 2006) (or spontaneous burning pain in humans (Schmelz et al., 2000)), followed by thermal and mechanical hypersensitivity that resolves within 3-7 days post-injection (Hankerd et al., 2021). As capsaicin induces both peripheral (Baumann et al., 1991) and central sensitization (Gazerani et al., 2005; La et al., 2017), secondary mechanical hypersensitivity is used commonly to infer the presence of capsaicin-induced central sensitization (La et al., 2017; LaMotte et al., 1991).

Another commonly used acute-injury pain model is the plantar incision model, which is a preclinical correlate of postoperative pain. Both deep incision through the plantar muscle and skin incision alone induce transient thermal hypersensitivity and secondary mechanical hypersensitivity that lasts 3-7 days (Brennan et al., 1996; Hankerd et al., 2021; Pogatzki & Raja, 2003; J. Xu & Brennan, 2010). Muscle incision was additionally demonstrated to induce spontaneous pain behaviors and induce a transient upregulation of Iba1, a marker of activated microglia (Ito et al., 1998), in the ipsilateral dorsal horn (Yoshiyama et al., 2021).

Nociplastic pain

As defined by the International Association for the Study of Pain (IASP), nociplastic pain is distinct from both nociceptive and neuropathic pain in that it 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). The term implies that (injury-induced) long-term changes in the nociceptive circuitry, such as peripheral and central sensitization, underlie this chronic pain. Nociplastic pain conditions, such as complex regional pain syndrome (CRPS) type I and chronic postoperative pain, predominately affect women (Melchior et al., 2016; Sandroni et al., 2003; Schug & Bruce, 2017; Soyama et al., 2015). As nociplastic pain conditions like CRPS have a complex etiology, patients symptomology is varied (Ott & Maihöfner, 2018). However, patients with CRPS commonly present with hypersensitivity to low-force (i.e., normally innocuous) mechanical hypersensitivity at and outside of the previously injured area (Gracely et al., 1992; Ott & Maihöfner, 2018). This secondary mechanical hypersensitivity has been attributed to central sensitization (Reimer et al., 2016).

Female gonadal hormones and sex differences in chronic pain

As briefly mentioned above, women are overrepresented among chronic pain conditions (Melchior et al., 2016) and tend to have longer lasting chronic pain (Heitkemper et al., 2003; Kindler et al., 2011; Paige et al., 2020; Tajerian et al., 2015; Vacca et al., 2016). Thus, studies are warranted to elucidate the biological factors contributing to sex disparities in the incidence, pathogenesis, and maintenance mechanisms of chronic pain (especially nociplastic pain).

Preclinical studies reporting sex differences generally agree that females tend to have longer lasting pain than their male counterparts (Paige et al., 2020; Tajerian et al., 2015; Vacca et al., 2016). It is noteworthy that the maintenance phase of hyperalgesic

priming, a form of long-term changes in the nociceptive circuitry for pain chronification, is prolonged in a female gonadal hormone-dependent manner (Paige et al., 2020). Female gonadal hormones also are critical mediators of sex-specific chronic pain mechanisms. Specifically, female gonadal hormones have been clearly demonstrated to inhibit microglial activation after neuropathic injury (Drew & Chavis, 2000; Lee et al., 2018; Wu et al., 2016) and to render the nociceptive system more readily sensitized by inflammatory agents (Avona, Mason, et al., 2021; Patil et al., 2019).

Of particular interest, estrogen is a well-known modulator of nociceptive circuitry, exerting site-dependent and dose-dependent pronociceptive (Claiborne et al., 2009; Kuhn et al., 2008; Li et al., 2009) and antinociceptive (Lee et al., 2018; Z. Z. Xu et al., 2019) effects. These divergent outcomes have been attributed to the distribution of specific estrogen receptors and their downstream signaling cascades. Notably, estrogen receptors α and the noncanonical membrane-bound G protein-coupled estrogen receptor (GPER) have been suggested to mediate the pronociceptive effects of estrogen, as agonism of estrogen receptors α (Y. Zhang et al., 2012) and GPER (Alvarez et al., 2014; An et al., 2014; Kuhn et al., 2008) induces or enhances nocifensive responses. These receptors have additionally been implicated as critical mediators of pain chronification, as they regulate hyperalgesic priming in females (Araldi et al., 2017; Ferrari et al., 2017). Accordingly, GPER's downstream targets have been implicated in the development of chronic pain (Ahmad & Ismail, 2002; Ji et al., 2009; Moy et al., 2017), including c-Fos (Hyder et al., 1991), mechanistic target of rapamycin complex 1 (mTORC1) (Wang et al., 2020), protein kinase C epsilon (PKCε) (Kuhn et al., 2008), and extracellular signal regulated kinase (ERK) (Filardo et al., 2000; Liverman et al., 2009).

SIGNIFICANCE

It is of interest and significance to understand how normally resolving pain induced by an acute injury becomes abnormally persistent despite the resolution of the injury (i.e., transitions into nociplastic pain). There is also an important gap in knowledge in terms of the female predominance in nociplastic pain conditions. Specifically, it is unclear if inherent female susceptibility or sexually dimorphic pain mechanisms contribute to the incidence of nociplastic pain, and if so, whether female gonadal hormones (particularly estrogen) play any role. Thus, with the goal of addressing these important questions, this project was designed to develop a clinically relevant murine model of nociplastic pain and investigate the mechanisms of the transition to, and the maintenance of, a nociplastic pain state in females. Based on these goals and previous studies demonstrating that normally innocuous postinjury stimulation can transiently prolong capsaicin-induced mechanical hypersensitivity (H. K. Kim et al., 2007; H. T. Kim et al., 2001), we hypothesized that 1) postinjury stimulation induces the transition to a nociplastic pain state in a sexually-dimorphic manner and 2) nociceptors at the previously injured area are rendered persistently active and maintain the nociplastic pain state in a female gonadal hormone-dependent manner.

A part of Chapter 2 (Materials and Methods) and the entire Chapter 3 were previously published by PAIN® with a co-first authorship with Kathleen E. McDonough (Hankerd et al., 2021).

Chapter 2: Materials and Methods

ANIMALS

Female and male C57BL/6N mice (5-10 weeks of age) were bred inhouse or purchased from Charles River (Houston, TX). Mice were housed in groups of up to five mice per cage under a 12-12 light-dark cycle and fed *ad libitum*. All experimental procedures were approved by the University of Texas Medical Branch's Institutional Animal Care and Use Committee and conducted in accordance with the National Institute of Health guidelines.

INTRADERMAL CAPSAICIN INJECTION

For behavioral experiments, freshly prepared capsaicin (3 μL, 0.1% in 10% ethanol, 10% Tween-20, and 80% saline; Sigma-Aldrich, St. Louis, MO) was injected intradermally into the base of the third and fourth toes of the plantar hind paw under 2.0-2.5% isoflurane anesthesia. For electrophysiological experiments using *ex vivo* skin-nerve preparations, two additional intradermal injections (3 μL each) were made to facilitate the sampling of afferents innervating the previously capsaicin-injected area: the first at the base of the hallux and the second at the medial hind paw (4-5 mm proximal to the primary injection).

PLANTAR INCISION

To limit potential nerve damage by deep muscle incision, we used a modification of the incision model in which the skin, but not deep muscle, is incised (Brennan et al., 1996; J. Xu & Brennan, 2010). Prior to surgery, buprenorphine-SR (0.5 mg/kg) was administered using a 30G needle. Under 2.5% isoflurane anesthesia, a single (~4 mm) incision was made

through the plantar hind paw from the base of the second to the fourth digits and suture-closed (9-0 microsurgical needle; Fine Science Tools, Foster City, CA).

POSTINJURY 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 hours after the injury (**Figure 1**). Postinjury thermal stimulation (30 or 40°C) was applied in the following manner: The distal half of 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 seconds every minute over a period of 10 minutes (10 times of 30 seconds in and 30 seconds 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. Similarly, in a subset of mice that received plantar incision, the incision area and surrounding tissues was stimulated with 40°C 23 hours post-incision as described above. For electrophysiological experiments using *ex vivo* skin-nerve preparations, the entire capsaicin-injected hind paw was similarly stimulated with 40°C warm water immersion.

BILATERAL OVARIECTOMY

Prior to surgery, buprenorphine-SR (1.0 mg/kg) was administered to female mice (aged 5-6 weeks). Under 2.5% isoflurane anesthesia, a single incision (~5 mm) was made into the medial dorsum skin. The fascia was separated from the muscle and an incision (~3 mm) was made into the muscle medial to each ovary. The ovary, ovarian fat, and attached superior uterine horn was resected and ligated; the remaining uterus (sans ovary) was

returned to the abdominal cavity. In sham-ovariectomized females, the ovary, ovarian adipose, and the uterine horn were withdrawn, identified, and returned to the abdominal cavity. After surgery, the skin was closed with stainless steel wound clips (9 mm; Becton, Dickinson and Company, Franklin Lakes, NJ). Mice were closely monitored after surgery to ensure a complete recovery.

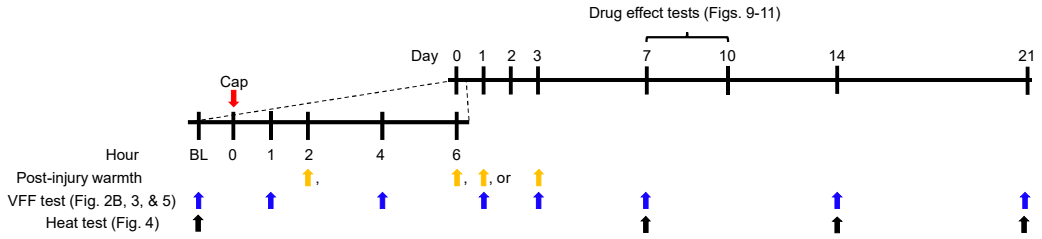

Figure 1: Timeline for behavioral experiments using capsaicin injection followed by postinjury thermal (warmth) stimulation paradigm.

After determining baseline (BL) behaviors, capsaicin (Cap, red arrow) was injected at the plantar side of 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 (blue arrows) and radiant heat tests (black arrows) were conducted at predetermined times in separate groups of mice. Effects of various drugs on persistent mechanical hypersensitivity were determined days 7 to 10 post-Cap.

ESTROGEN RECONSTITUTION

Seven or eleven days after bilateral ovariectomy (**Figure 14**), a subset of ovariectomized females were implanted with an osmotic minipump (#1004; Alzet, Cupertino, CA, USA) containing 17β-estradiol (18 µg/mL in 10% DMSO, 10% Tween-20, and 80% saline, equivalent to 1.98 ng/day; Sigma-Aldrich). Serum 17β-estradiol was quantified using an enzyme-linked immunosorbent assay (ELISA) (Calbiotech, El Cajon, CA) per manufacturer instructions.

MECHANICAL SENSITIVITY TEST

Mice were habituated to behavioral test conditions (including experimenters) for 4 days as previously described (La et al., 2017) before 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 before 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% to 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 et al., 2013)) or *in vivo* from C3H/HeJ mice (the interquartile range 10-25 mN (Cain et al., 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 postinjury stimulation, we stimulated the area outside the injury (4-5 mm proximal to the injured area; mid-hind paw) with a 0.98 mN von Frey filament. Mechanical hypersensitivity is known to develop outside the injured area (commonly called secondary mechanical hypersensitivity) because of the injury-induced sensitization of nociceptive system at a central level. The percentage of withdrawal responses out of 10 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 the injured area, which, therefore, could confound the "postinjury stimulation of the injured area" paradigm when used before capsaicin-induced thermal hypersensitivity substantially abates (see **Figure 6**). Therefore, except for the experiment determining the resolution time course of capsaicin-induced thermal hypersensitivity (**Figure 2**) we performed this radiant heat test at baseline, and after persistent mechanical hypersensitivity was established.

EVANS BLUE EXTRAVASATION

Under 2% isoflurane anesthesia, Evans Blue (50 mg/mL; Sigma-Aldrich) was intravenously administered (50 mg/kg) through the tail vein either 2 hours, 1 day, or 7 days after capsaicin injection with or without 40°C water immersion at 2 hours post-capsaicin; for 2 hours post-capsaicin time point data in the water immersion group, Evans Blue injection was performed immediately after the immersion. Thirty minutes after Evans Blue injection, mice were perfused with saline, and glabrous skin samples (~2 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 hours. Evans Blue dye deposits were extracted in formamide (16 mL per 1.0 g dry weight tissue; Sigma-Aldrich) at 37°C for 72 hours. The concentration of Evans Blue was quantified using a NanoDrop 2000C (Thermo Fisher, Waltham, MA) and analyzed as described in the literature (Krzyzanowska et al., 2010).

QUANTIFICATION OF PROINFLAMMATORY CYTOKINE GENE TRANSCRIPTS

One day or 7 days after capsaicin injection, skin samples (~2 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 and then transferred to an Eppendorf tube containing TRIzol reagent (Thermo Fisher) with 1 mg/mL collagenase type I (125 U/mg, Gibco, Waltham, MA). Using a bead mill homogenizer with micro glass beads (0.5 mm; Biospecs Products, Bartlesville, OK), tissue was processed for 10 to 15 cycles of 120 seconds at speed 5. The supernatant was isolated, and 400 mL pheno-chloroform (Amresco, Solon, OH) per 1.0 mL TRIzol reagent was added. After centrifuging the mixture at 12,000 *x g* for 15 minutes, the supernatant was transferred to a fresh Eppendorf tube containing an equal volume of isopropanol, incubated on wet ice for 10 minutes, and centrifuged at 10,000 *x g* for 10 minutes. Finally, the RNA pellet was rinsed 3 times with 1.0 mL 70% ethanol and centrifuged at 5,000 *x g* for 5 minutes before resuspension in nuclease-free water. RNA quality and purity were checked using a NanoDrop 2000C (Thermo Scientific) before 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) was mixed with 10 ng of cDNA and β-2-microglobulin primer (forward: 5'-TGGTCTTTCTGGTGCT TGTC-3', 100 mM; reverse: 5'-GCAGTTCAGTATGTTCGGCT- 3', 100 mM), IL-1β primer (forward: 5'-CTGGTGTGTGACGTTCC CATTA-3', 100 mM; reverse: 5'-CCACAGCACGAGGCTTT-3', 100 mM), IL-6 primer (forward: 5'-AAGAACAAAGCCAGAGTC CTTC-3', 300 mM; reverse: 5'-TAGGAGAGCATTGGAAATTG GG-3', 300 mM), or TNF-α primer (forward: 5'-CCCTCACACT CACAAACCAC-3', 300 mM; reverse: 5'-TTTGAGATCCATGCC

GTTGG-3', 300 mM). qPCR was conducted at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 55.6°C for 30 seconds, and 60°C for 30 seconds. Single amplicon was confirmed by the 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.

DRUG ADMINISTRATION

All drugs were administered using a 30G needle under 2.0-2.5% isoflurane anesthesia. Complete peripheral afferent silencing at the previously capsaicin-injected area was performed 7 to 10 days after capsaicin plus postinjury stimulation by intradermally injecting normal bupivacaine (0.75% in saline; Fluka, Buchs, Switzerland) or saline. Gabapentin (100 mg/kg; Spectrum Chemical Mfg Corporation, New Brunswick, NJ) or morphine (5 mg/kg; Westward, Eatontown, NJ) or saline (0.9%; Baxter Healthcare Corporation) was injected intraperitoneally 7 to 10 days after capsaicin plus postinjury stimulation. In experiments assessing the contribution of the G Protein-coupled Estrogen Receptor (GPER) to persistent mechanical hypersensitivity, G-36 (50 μg/kg in 10% DMSO, 10% Tween-20, and 80% saline; Cayman Chemical, Ann Arbor, MI) or vehicle was injected subcutaneously every 24 hours either 1) immediately following postinjury stimulation (day 0) through day 3 post-capsaicin injection or 2) from day 4 post-capsaicin

injection through day 10 post-capsaicin injection (after each timepoint, **Figure 14**). Silencing specific afferent populations at the previously capsaicin-injected area was performed 7 to 10 days after capsaicin plus postinjury stimulation by intradermally injecting lidocaine N-ethyl bromide (QX-314, 2.0%, Sigma Aldrich) alone or in conjunction with capsaicin (0.1%, Sigma-Aldrich), allyl isothiocyanate (AITC, 30 μM, Fluka), or flagellin (0.9 ng, InvivoGen, San Diego, CA, USA). The TRPA1 receptor antagonism at the previously capsaicin-injected area was performed 7 to 10 days after capsaicin plus postinjury stimulation by intradermally injecting HC-030031 (30 μM in 10% DMSO, 10% Tween-20, and 80% saline; Calbiochem, San Diego, CA).

EX VIVO SKIN-NERVE PREPARATION ELECTROPHYSIOLOGY

Skin-nerve preparations were prepared as previously described (Shim et al., 2019). Specifically, the hind paw glabrous skin and the attached tibial nerve were removed 7 to 10 days after capsaicin (acute pain model) or capsaicin plus postinjury thermal (40°C) stimulation (nociplastic pain model). The skin was transferred corium-side up to a bath of artificial interstitial fluid (131.1 mM sodium chloride, 9.6 mM sodium gluconate, 5.6 mM glucose monohydrate, 7.6 mM sucrose, 10.0 mM HEPES, 1.32 μM magnesium sulfate, 3.5 μM potassium chloride, 2.0 μM sodium phosphate monohydrate, and 2.7 μM calcium chloride; pH 7.40; 30ºC). The mechanosensitivity and innervation area of small bundles of units were detected by stimulating the hind paw skin with a blunt glass probe. After 10 minutes of recording spontaneous activity, ramp mechanical stimulation (0-200 mN over 20 seconds, Aurora Scientific Instruments, Aurora, ON, CA) was applied to the receptive field of the nerve terminals. At the end of each recording, afferents were phenotyped by sequentially applying 1) electrical stimulation (World Precision Instruments, Saratosa, FL,

USA) and 2) topical artificial interstitial fluid followed by AITC (30 μM) to the afferent terminals to determine conduction velocity and responsiveness to AITC, respectively. Units with conduction velocities ≤1.3 m/s were classified as C-fibers, ≥13.0 m/s Aβ fibers, and Aδ fibers if their conduction velocity was >1.3 m/s and <13.0 m/s (Cain et al., 2001). Units were identified as AITC-responsive if they fired burst action potentials when AITC (30 μM, Fluka), but not artificial interstitial fluid, was topically applied. An additional subset of units, whose baseline firing rate doubled when AITC was topically applied, were also considered AITC-responsive. Recordings from naïve mice and the contralateral hind paw were pooled as controls.

STATISTICS

Statistical tests were performed using SPSS (ver. 25, IBM, NY, USA) or Graphpad Prism (ver. 8, Graphpad, CA).

Behavior data

Thermal sensitivity data measuring paw withdrawal latency at multiple time points was analyzed using linear mixed model (LMM) with the first-order autoregressive (AR1) covariance structure for repeated measures (Time) followed by Sidak multiple comparison tests. Data are presented as mean ± SD or individual values.

Mechanical sensitivity data was analyzed using generalized linear mixed model (GLMM) with the logit link function for binomial distribution and the AR1 covariance structure for repeated measures (Group x Time [repeated]). Degrees of freedom were allowed to vary using Welch–Satterthwaite function. Multiple comparisons between timepoints (as compared to the baseline or pre-drug timepoint) within each group were

made using sequential Sidak procedure. Because the percentage of nocifensive withdrawal responses (of 10 trials) is not normally distributed, it is presented as median with interquartile range (IQR) at each timepoint.

Curve fitting

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 hours post-capsaicin (ΔL_{2h}) in individual mice. Capsaicin-induced mechanical hypersensitivity was similarly analyzed, with the difference between % withdrawal at baseline and each time point (ΔW_t) normalized to the peak difference at 1 hour post-capsaicin (ΔW_{1h}) 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.

Evans Blue extravasation

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). Data are presented as individual values.

Quantification of proinflammatory cytokine gene transcripts

The gene expression levels of proinflammatory cytokine transcripts 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). Data are presented as individual values.

Ex vivo skin nerve preparation electrophysiology

Individual units within each recording were distinguished using principal component analysis (Spike 2, ver. 5.21, Cambridge Electrical Design, Cambridge UK). Analyses were confined to each hormonal (i.e., gonad-intact females, ovariectomized females, and males) group; within each hormonal group, AITC-responders and AITC-nonresponders were analyzed in the same statistical test. The proportion of spontaneously active units was analyzed using a GLMM for binomial logistic regression. Mean spontaneous firing frequency of spontaneously active units and mechanical threshold were log transformed to resolve heteroscedasticity. Mean spontaneous firing frequency and mechanical threshold were analyzed using a GLMM (Group [afferent nested within animal] x Treatment x AITC responsiveness) with the identity link function for each hormonal group. Multiple comparisons were performed using a sequential Sidak procedure. The total number of action potentials in response to ramp stimulation were analyzed using a GLMM with a log link function for Poisson distribution (Group [afferent nested within animal] x Treatment) and sequential Sidak procedure for multiple comparisons. Data are presented as individual values.

Chapter 3: Postinjury stimulation triggers the transition to a nociplastic pain state[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 CRPS type I and chronic postsurgical pain, pain can persist long after the inciting injury has healed, without apparent organic abnormalities underlying the pain (Mccabe & Blake, 2008; Steegers et al., 2008). This type of chronic pain, recently termed "nociplastic pain" by the International Association for the Study of Pain taskforce, is a unique category of chronic pain disorders distinct from idiopathic and neuropathic pain (Kosek et al., 2016). In such disorders, nociplastic pain arises from altered nociception in the absence of tissue damage or overt neuropathy directly driving the pain (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 et al., 2006; Melchior et al., 2016; Sandroni et al., 2003). The female 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

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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 (Jiao et al., 2015), postinfectious irritable bowel syndrome, and chronic postsurgical pain, potential or obvious inciting injuries (trauma, infection, etc.) could be identified. Because 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.

Regarding such transition, chronic postsurgical pain provides valuable information about risk factors. Surgery being considered a tissue injury, clinical findings indicate that the magnitude of postsurgical (i.e., postinjury) pain itself 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 postinjury 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 postinjury stimulation in both male and female mice, without apparent persistent tissue damage, and that this prolonged mechanical hypersensitivity is maintained by sexually dimorphic mechanisms.

RESULTS

Intraplantar capsaicin injection induces mechanical and thermal hypersensitivity that resolves slower in females than in males

Before developing a nociplastic pain animal model using a postinjury thermal stimulation to increase postinjury 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 hours post-capsaicin being set as the 100% peak for each mouse) revealed slower resolution in females than in males (**Figure 2B**: 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). We also noted differences in the resolution of capsaicin-induced mechanical hypersensitivity. Although both sexes showed a similar degree of mechanical hypersensitivity at 1 hour post-capsaicin and a gradual decrease over the following 3 days, females manifested significantly greater mechanical hypersensitivity than males at 1 day post-capsaicin (**Figure 3A**: t(105) =2.60 by sequential Sidak test, P=0.011). Curve fitting of the resolution of capsaicin-induced mechanical hypersensitivity similarly indicated a slower resolution of capsaicin-induced mechanical hypersensitivity in females than males (**Figure 3B**: R^2 = 0.75 for females, 0.66 for males; F(2,115) =11.8, P <0.001; the tau was 2.3 days for females (95% CI: 1.5-3.5 days) and 0.5 day for males (95% CI: 0.3-0.7 day)).

Together, this data suggests that capsaicin-induced thermal and mechanical hypersensitivity subsides slower in females.

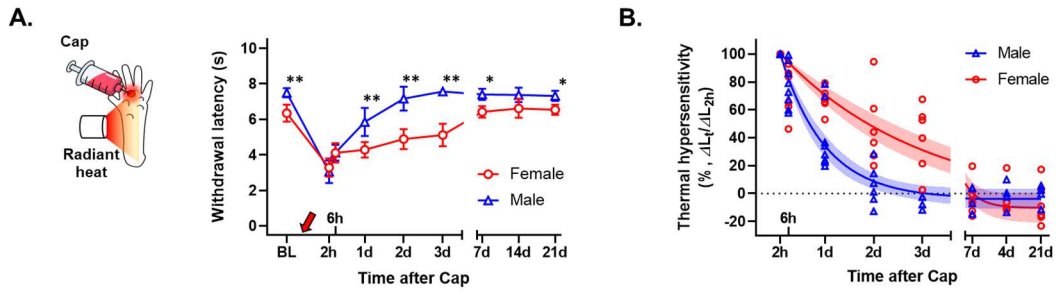

Figure 2: Sex difference in capsaicin-induced thermal hypersensitivity.

(**A**) Mice developed thermal hypersensitivity after intraplantar capsaicin (Cap, red arrow) injection, showing decreased latency to withdraw from radiant heat. Females showed a slower recovery to the baseline (BL) level than males. *P <0.05, **P <0.01 between males and females by the Sidak multiple comparison test. Data are presented as mean ± standard deviation. (**B**) Curve fitting of Cap-induced thermal hypersensitivity revealed slower resolution of the hypersensitivity in females than in males. ΔL_t, the difference between withdrawal latencies at baseline and each time point; ΔL_{2h}, 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.

Postinjury stimulation prolongs 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 seconds per minute for 10 minutes at 2 hours 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). Although 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 (**Figure 4**); the capsaicin plus 40°C group showed greater mechanical hypersensitivity than capsaicin control in both sexes from day 1 (male) or day 3 (female) 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).

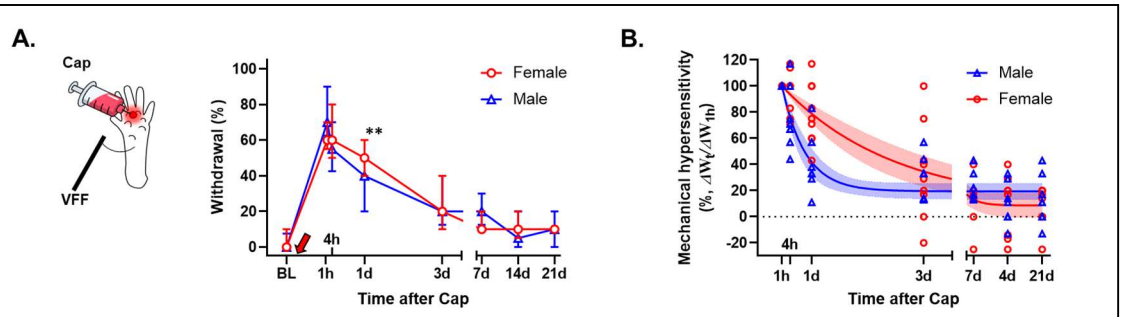

Figure 3: Sex difference in capsaicin-induced mechanical hypersensitivity.

(**A**) Mice developed mechanical hypersensitivity after intraplantar capsaicin (Cap, 0.1%, red arrow) 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 showed greater mechanical hypersensitivity at 1 day post-capsaicin than males. **P <0.01 between males and females by the Sidak multiple comparison test. Data are presented as median with interquartile range. (**B**) Curve fitting of capsaicin (Cap)-induced mechanical hypersensitivity revealed slower resolution of the hypersensitivity in females than in males. ΔW_t, the difference in the percentage withdrawal between baseline (BL) and each time point; ΔW_{1h}, the peak difference (100%) at 2 hr post-Cap. The shaded area indicates 95% confidence interval. Males: n = 8. Females: n = 9. BL, baseline.

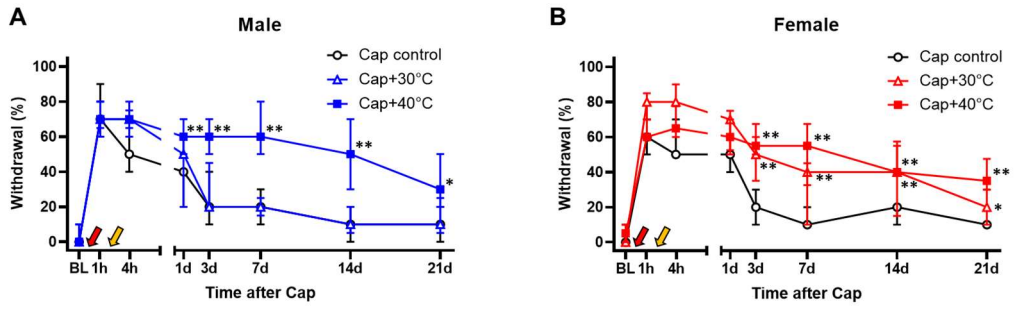

Figure 4: Chronification of capsaicin-induced mechanical hypersensitivity by postinjury thermal stimulation.

In both (**A**) males and (**B**) females, 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. When 30°C water was used instead of 40°C water, (**A**) females, but not (**B**) males, showed a significant chronification. *P <0.05, **P <0.01 vs Cap control by sequential Sidak multiple comparison tests. Data are presented as median with interquartile range. Males: n = 8 in Cap control, n = 9 in Cap+30°C, and n = 11 in Cap+40°C. Females: n = 9 in Cap control, n = 9 in Cap+30°C, and n = 8 in Cap+40°C. BL, baseline.

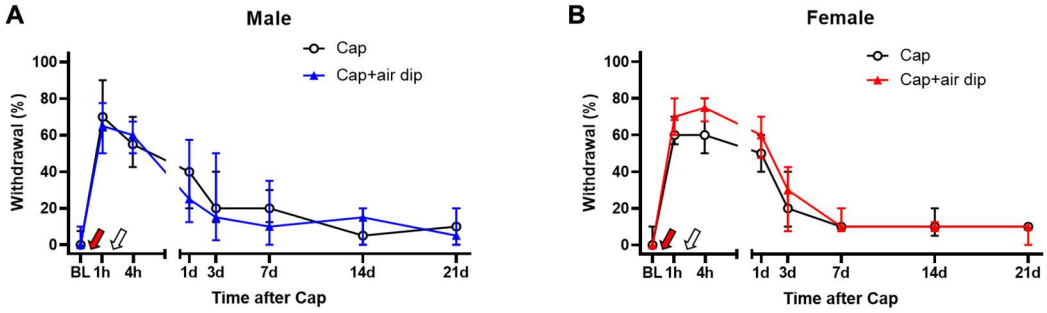

Figure 5: Capsaicin-induced mechanical hypersensitivity is not affected by hind paw immersing motion itself.

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 median with interquartile range. 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.

We subsequently determined whether the immersion movement of the capsaicin-injected hind paw for postinjury thermal stimulation itself was contributing to this prolong capsaicin-induced mechanical hypersensitivity in the area outside the capsaicin injection site. This "air immersion" was performed 2 hours after capsaicin injection. As shown in **Figure 5**, 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.

We next asked whether chronification of capsaicin-induced mechanical hypersensitivity would depend on the intensity of postinjury thermal stimulation. When the capsaicin-injected hind paw was stimulated with 30°C water at 2 hours 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 postinjury thermal stimulation for all future experiments. Of note, capsaicin-induced thermal hypersensitivity was not prolonged by the 40°C postinjury stimulation; the latency to withdraw from radiant heat at 7 to 21 days post-capsaicin did not differ from the baseline values in both sexes (**Figure 6**). Therefore, we focused our later studies on chronification of mechanical hypersensitivity.

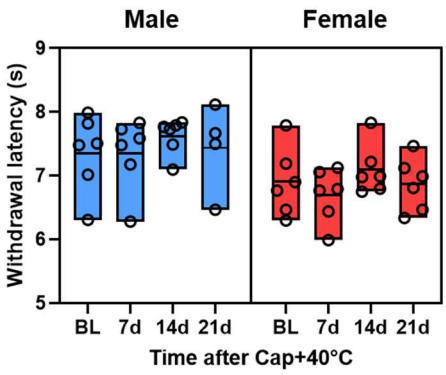

Figure 6: Chronification of capsaicin-induced thermal hypersensitivity does not occur by 40°C postinjury stimulation.

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. The bottom, middle line, and top of each bar indicate the minimum, mean, and maximum values in each group, respectively. Males: n = 6 until day 14 and n = 4 at day 21. Females, n = 6 throughout.

In our modeling approach, postinjury 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 postsurgical (i.e., postinjury) pain. Therefore, in our experimental design using capsaicin as an experimental injury, we hypothesized that the effect of postinjury 40°C stimulation, which serves to increase postinjury 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 postinjury 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 hours post-capsaicin). Because the thermal hypersensitivity resolves differentially between sexes as shown in **Figure 2B**, we chose sex-specific time

points for the 2 abating phases: 6 hours vs 1 day post-capsaicin for males and 1 day vs 3 day 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 before the postinjury stimulation. When capsaicin-induced thermal hypersensitivity abated to approximately 70% (i.e., 6 hours 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 (**Figure 7A and B**). When the thermal hypersensitivity subsided to approximately 35% (i.e., 1 day in males and 3 day in females), mechanical hypersensitivity was not prolonged in males (**Figure 7C**); however, in females, the hypersensitivity was still significantly increased at time points later than day 3 (**Figure 7D**; note that the postinjury stimulation was applied 30 minutes before the behavioral tests on day 3). These data suggest that the magnitude of capsaicin-induced thermal hypersensitivity at the time of postinjury thermal stimulation is predictive of mechanical hypersensitivity chronification, and females have a wider timeframe than males, in which postinjury stimulation can trigger such chronification.

We next investigated the potential limitation of the ability for postinjury 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 hours post-incision (i.e., 1 hour before behavioral tests on day 1). We found that the incision-induced mechanical hypersensitivity was significantly prolonged as shown in **Figure 8A and B**. These data indicated that mechanical hypersensitivity chronification by postinjury thermal stimulation is not restricted to the capsaicin model. Observing that these different modeling paradigms commonly demonstrate that postinjury stimulation prolongs

postinjury mechanical hypersensitivity beyond the normal resolution time, we chose to use capsaicin injection followed by 40°C thermal stimulation at 2 hours post-capsaicin (capsaicin plus 40°C) throughout the rest of this study.

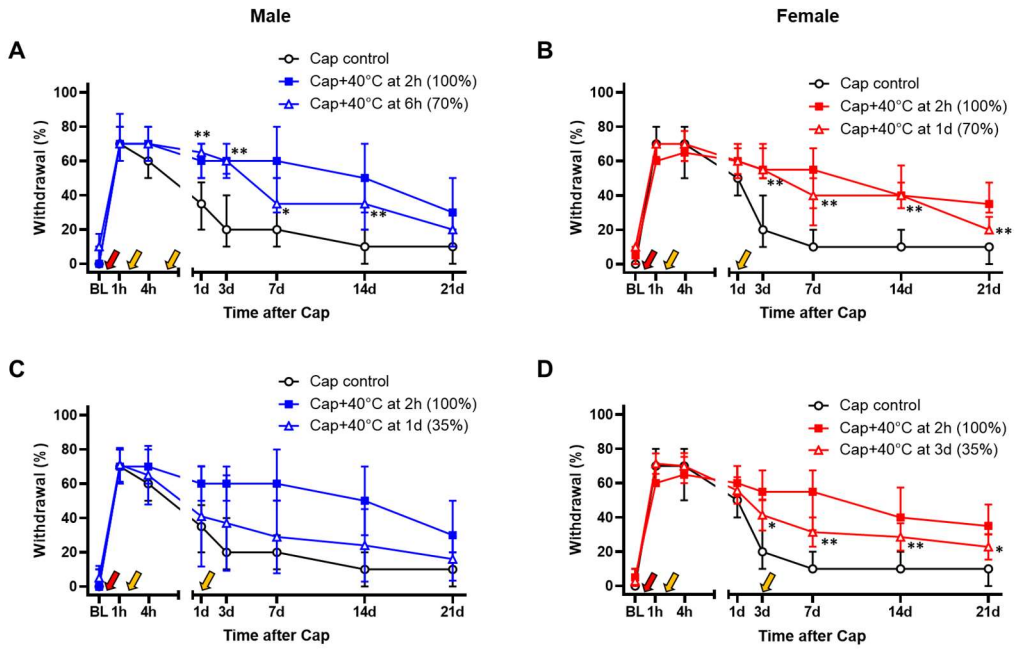

Figure 7: Magnitude of capsaicin-induced thermal hypersensitivity at the time of postinjury thermal stimulation is predictive of mechanical hypersensitivity chronification.

In both (**A**) males and (**B**) females, 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), (**D**) females still showed significantly greater mechanical hypersensitivity at time points ≥ day 3 post-Cap, while 40°C postinjury stimulation was insufficient to produce chronification of capsaicin-induced mechanical hypersensitivity in (**C**) males. *P <0.05, **P <0.01 vs Cap control by sequential Sidak multiple comparison tests. Data are presented as median with interquartile range. *P <0.05, **P <0.01 vs Cap control by sequential Sidak multiple comparison tests 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. As the same Cap+40°C at 2 hours group from **Figure 4** was used for statistical comparisons here, differences between Cap+40°C at 2 hours and all the other groups in **Figures 7** were analyzed in the same statistical test. BL, baseline.

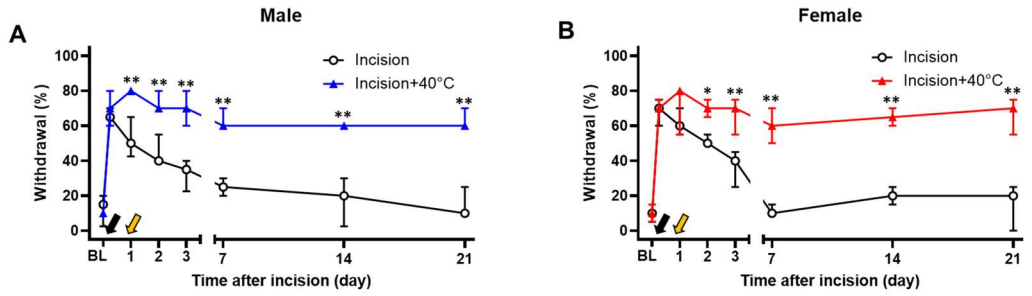

Figure 8: Confirmation of mechanical hypersensitivity chronification using different modeling paradigms.

When the hind paw was immersed in 40°C water (30 seconds/minute for 10 minutes, yellow arrow) 23 hours after plantar incision (black arrow), the incision-induced mechanical hypersensitivity was significantly prolonged in both (**A**) males and (**B**) females. **P <0.01 vs corresponding control. Data are presented as median with interquartile range. Males: n = 4 in Incision and n = 7 in Incision+40°C. Females: n = 5 in each group. BL, baseline.

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

Having found that postinjury stimulation of the capsaicin-injected paw at 2 hours 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 et al., 1968; Lin et al., 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 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 with the corresponding contralateral area (**Figure 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 hours 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.

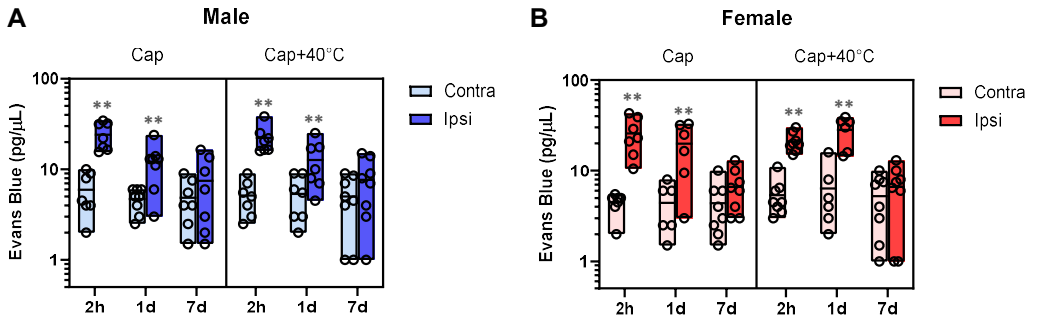

Figure 9: No evidence of increased vascular leakage at the capsaicin-injected area 7 days after capsaicin plus 40°C postinjury stimulation.

In both (**A**) males and (**B**) females, 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.

In addition, we quantified proinflammatory cytokine gene transcripts at the capsaicin injection area. As shown in **Figure 10**, tissue contents of IL-1b 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 was also elevated in the capsaicin-injected area. The TNF-a 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 note, statistically significant increases in the quantities of IL-6 ($t(23.9) = 4.44$, $P=0.001$) and TNF-a mRNAs ($t(23.7) = 3.82$, $P=0.005$) were detected only in the 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.

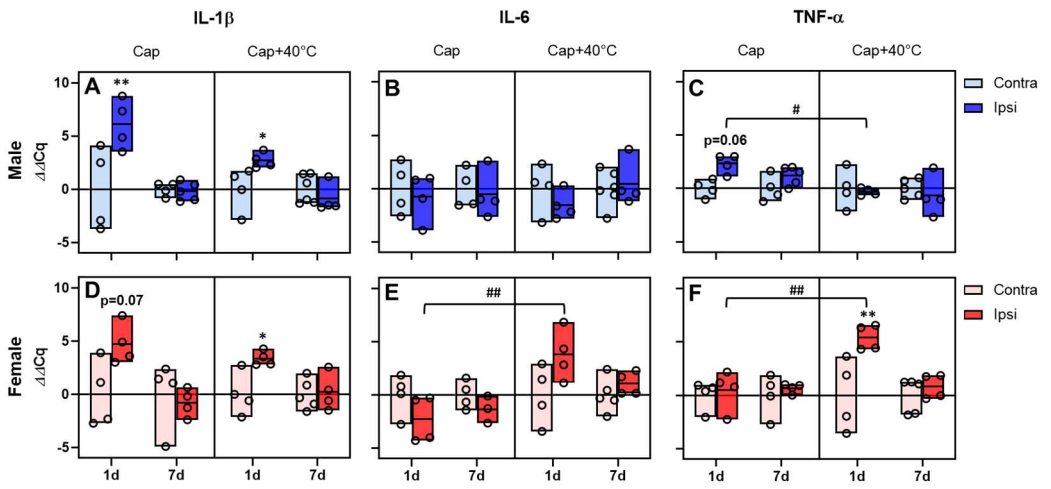

Figure 10: No evidence of increased gene expression of proinflammatory cytokines at the capsaicin-injected area 7 days after capsaicin plus 40°C postinjury stimulation.

In both (**A**) males and (**D**) females, 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 (**B and E**) IL-6 and (**C and F**) tumor necrosis factor (TNF)-α also did not differ between contralateral and ipsilateral sides. In female Cap+40°C group, (**E**) IL-6 and (**F**) TNF-α mRNAs were significantly increased in the ipsilateral side at 24 hours post-capsaicin, compared with those in female Cap control. 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. *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. 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. 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.

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 (Baumann et al., 1991; 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 **Figure 11**, both morphine and gabapentin immediately and robustly inhibited the persistent mechanical hypersensitivity present at days 7 to 10 post-capsaicin in both males and females. These results also indicated the 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) 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 (Sang et al., 1996). 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 to 10 days post-capsaicin to silence afferents innervating the area. As shown in **Figure 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.

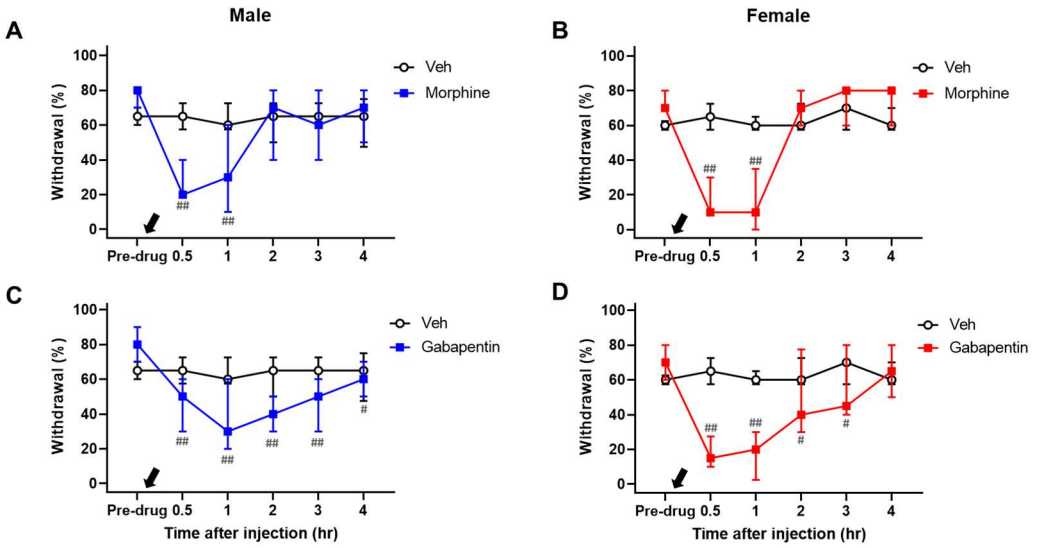

Figure 11: Alleviation of persistent mechanical hypersensitivity by pain medications.

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. #P <0.05, ##P <0.01 vs pre-drug level in each group by sequential Sidak multiple comparison tests. Data are presented as median with interquartile range. 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.

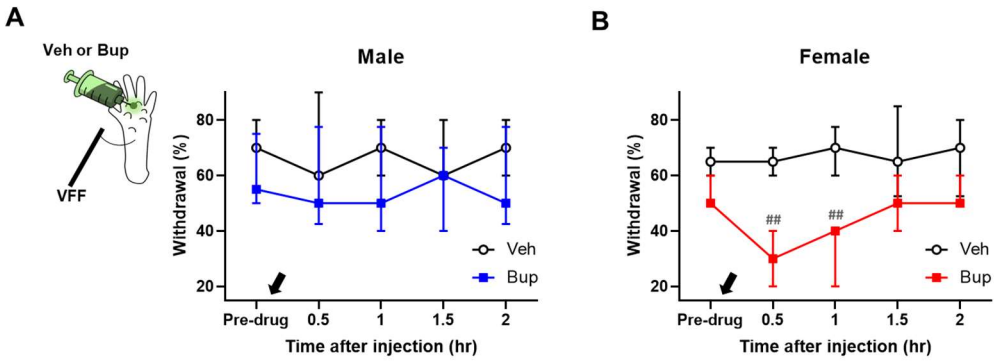

Figure 12: Female-specific alleviation of persistent mechanical hypersensitivity by local anesthesia of previously capsaicin-injected area.

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 (**A**) males, it significantly alleviated the hypersensitivity in (**B**) females at 7 to 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. Data are presented as median with interquartile range. Males: n = 7 in vehicle (Veh) and n = 8 in Bup. Females: n = 8 in Veh and n = 7 in Bup. VFF, von Frey filament.

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 to 10 days post-capsaicin. We observed that Mac-1-saporin treatment significantly attenuated persistent mechanical hypersensitivity in

males, but not in females (**Figure 13A and B**). We immunostained spinal cord samples from these mice and quantified the immunoreactivity of Iba1, a protein upregulated in activated microglia (Ito et al., 1998). As shown in **Figure 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. Such a difference in Iba1-immunoreactivity between ipsilateral and contralateral 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 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 maintains the persistent mechanical hypersensitivity.

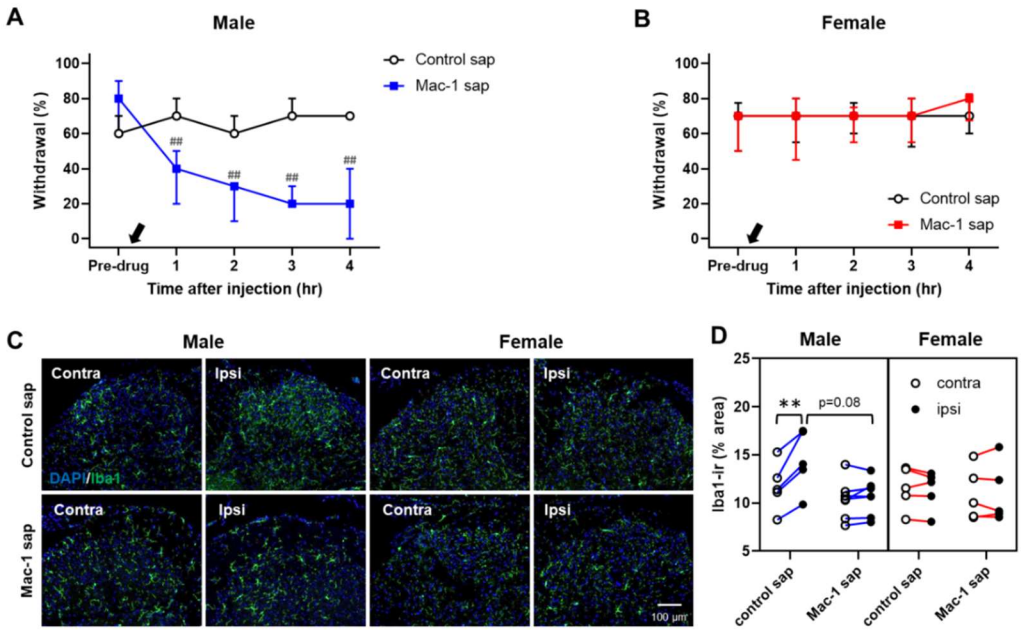

Figure 13: Male-specific alleviation of persistent mechanical hypersensitivity by inhibition of spinal microglia.

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 (**A**) males but not in (**B**) females at 7 to 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. Data are presented as median with interquartile range. (**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); the increased Iba1 immunoreactivity (Iba1-ir) in the male nociplastic pain model was effectively decreased by intrathecal Mac-1-sap. No difference was detected between either dorsal horn sides or treatment groups in the female nociplastic pain model. **P < 0.01 by Bonferroni tests for 4 pairwise comparisons in each sex. Males: n = 7 in each group. Females: n = 8 in control sap and n = 9 in Mac-1-sap.

DISCUSSION

In this study, we introduce a novel mouse model to facilitate elucidation identification of mechanisms for the transition to and maintenance of a nociplastic pain state. In this model, postinjury thermal (40°C) stimulation 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 because of chronic inflammation.

Similar to the hyperalgesic priming (type I) model that has been used for studying mechanisms of pain chronification (Parada et al., 2005), we also used a paradigm of an initial injury (to "prime/sensitize" the nociceptive system) followed by a postinjury 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 postinjury stimulation, as the postinjury 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 postinjury stimulation (most commonly an injection of inflammatory mediators) given after the initial injury-induced hypersensitivity completely resolves. In addition, although 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 postinjury 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 patients with chronic pain can be stratified by their sensory profiles (e.g., mechanical hyperalgesia-predominant 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 postinjury stimulation would be a factor for such a "circuit specific" chronification of nociceptive system sensitization. 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 & 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). Thus, it remains to be investigated whether this additional model would also manifest a circuit-specific chronification of nociceptive system sensitization.

Clinically, women are disproportionately affected by nociplastic pain (Melchior et al., 2016; Sandroni et al., 2003; Schug & Bruce, 2017; Steegers et al., 2008). 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 postinjury 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 postinjury stimulation can trigger pain chronification. Therefore, if any postinjury 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 the sensitized nociceptive system. As sex differences in capsaicin-induced hypersensitivity (Barrett et al., 2003) and nociceptor priming in the form of hyperalgesic priming are female gonadal hormone dependent (Paige et al., 2020), it would be interesting to assess whether the removal of female gonadal hormones makes females less susceptible to pain chronification.

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 complex regional pain syndrome 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 et al., 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 the female capsaicin plus postinjury thermal stimulation group than in capsaicin controls. Considering that peripheral injection of IL-6 or TNF-α induces hyperalgesic priming in nociceptors (Dina et al., 2008; Joseph et al., 2003), it could be that these cytokines (elevated by postinjury 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. In addition, 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 & Malcangio, 2014; Inoue et al., 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 postinjury 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 postinjury stimulation.

In conclusion, we have developed a novel mouse model that meets the criteria of International Association for the Study of Pain'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.

Chapter 4: Peripherally maintained nociplastic pain mechanisms is female gonadal hormone-dependent

INTRODUCTION

Nociplastic pain is female-predominant chronic pain (Melchior et al., 2016; Sandroni et al., 2003; Soyama et al., 2015) arising from altered nociception in the absence of tissue damage or overt neuropathy directly driving the pain (Kosek et al., 2016). Though the incidence of these conditions skews towards women, clinical studies on whether female gonadal hormones confer susceptibility and/or are mechanistic drivers of nociplastic pain are equivocal (Buryanov et al., 2017; de Mos et al., 2009). For instance, though female overrepresentation among Complex Regional Pain Syndrome (CRPS) type I, a nociplastic pain condition, arises after puberty, this sex disparity persists beyond menopause (Sandroni et al., 2003), raising the question of whether female gonadal hormones are necessary for and/or act as mechanistic drivers of the development of nociplastic pain in women.

In the previous chapter, we introduced a murine model in which postinjury stimulation (at normally innocuous intensity) of an injured area induces the transition from acute injury-induced pain to nociplastic pain that manifests as persistent (i.e., nociplastic) mechanical hypersensitivity outside the previously injured area without apparent ongoing tissue damage (Hankerd et al., 2021). Of note, this nociplastic mechanical hypersensitivity is maintained by sexually dimorphic mechanisms. In female mice, but not in male mice, the hypersensitivity is maintained by ongoing afferent activity at the previously injured area, which is reminiscent of the clinical findings in CRPS patients (Gracely et al., 1992), indicating that the nociplastic pain state is peripherally maintained in female mice.

Using this murine model of nociplastic pain, here we investigated whether female gonadal hormones are critical for the development of the nociplastic pain state in females. We also identified the afferent type whose ongoing activity at the previously injured area maintains nociplastic mechanical hypersensitivity in females. Lastly, we determined whether this ongoing afferent activity was female gonadal hormone-dependent.

RESULTS

Female gonadal hormones are not required for postinjury stimulation to prolong capsaicin-induced mechanical hypersensitivity

Two weeks after sham-ovariectomy or bilateral ovariectomy, 0.1% capsaicin was injected intradermally as an experimental injury. In our previous study, this dose of capsaicin induces transient mechanical hypersensitivity outside of the injection area that resolves within 7 days in males and gonad-intact females (Hankerd et al., 2021). As shown in **Figure 15**, in both sham-ovariectomized (Sham-OVX) and ovariectomized (OVX) females, capsaicin-induced mechanical hypersensitivity outside of the injection area gradually resolved over 7 days. Stimulation of the capsaicin-injected area by intermittently immersing the capsaicin injected area in warm (40°C) water for 10 minutes significantly prolonged capsaicin-induced mechanical hypersensitivity in both sham-OVX and OVX females such that it persisted up to 21 days post-capsaicin (**Figure 15**: Cap vs. Cap+40°C at day 21 post-capsaicin: $t(95) = 4.027$, $P < 0.001$ by sequential Sidak test in Sham-OVX; $t(52) = 3.115$, $P = 0.003$ by sequential Sidak test in OVX). This result indicated that female gonadal hormones are not required for postinjury stimulation to make capsaicin-induced mechanical hypersensitivity persistent in females.

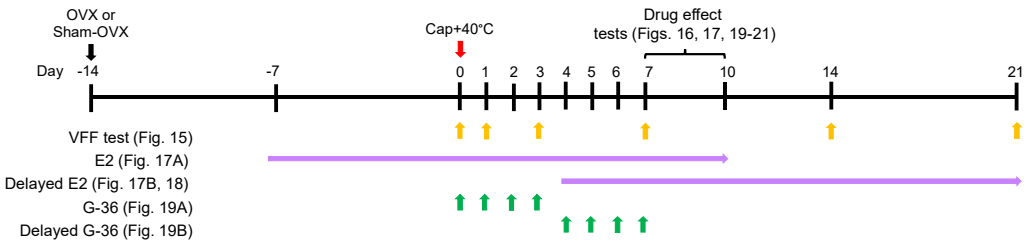

Figure 14: Timeline for behavioral experiments examining female-specific mechanisms of nociplastic mechanical hypersensitivity

Two weeks before capsaicin plus 40°C postinjury stimulation (Cap+40°C, red arrow), female mice were sham-ovariectomized (Sham-OVX, black arrow) or ovariectomized (OVX, black arrow). A subset of OVX females were implanted with osmotic mini pumps containing 17β-estradiol (E2, purple arrows) on day -7, whereas OVX females receiving delayed E2 were implanted with mini pumps 4 days post-Cap+40°C. In another series of experiments, gonad-intact female mice received the estrogen receptor GPER antagonist G-36 (green arrows) either during the transition phase (day 0 to 3 post- capsaicin+40°C) or after the establishment of a nociplastic pain state (day 4 to 7 post-Cap+40°C). The effect of various drugs on nociplastic mechanical hypersensitivity were determined days 7 to 10 post-Cap+40°C. Mechanical sensitivity was assessed using a von Frey filament (VFF, blue arrows).

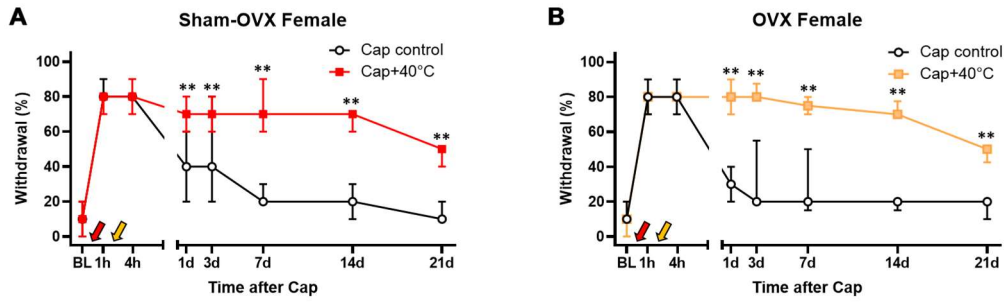

Figure 15: Ovariectomy does not prevent the transition to a nociplastic pain state by postinjury stimulation

In both (**A**) sham-ovariectomized (Sham-OVX) females and (**B**) ovariectomized (OVX) females, postinjury thermal (40°C, yellow arrow) stimulation of a capsaicin (Cap, 0.1%, red arrow)-injected area made capsaicin-induced mechanical hypersensitivity persistent. **P<0.01, Cap control vs Cap+40°C by sequential Sidak test. Data are presented as median with interquartile range. Sham-OVX females: n = 7 per group. OVX females: n = 7 per group until 72 hours, after which n = 5 in Cap control and n = 4 in Cap+40°C.

Female gonadal hormones are required to make nociplastic mechanical hypersensitivity dependent upon ongoing afferent input

Previously, we found that postinjury stimulation triggers the transition to the nociplastic pain state in both males and females but that this nociplastic mechanical hypersensitivity is peripherally maintained (by ongoing nerve activity at the previously capsaicin-injected area) only in females. Thus, we next investigated whether OVX females have a peripherally maintained nociplastic pain state, like gonad-intact females. To this end, afferents at the previously capsaicin-injected area were silenced using the local anesthetic bupivacaine 7 to 10 days after capsaicin plus postinjury thermal (40ºC) stimulation (henceforth termed "capsaicin+40ºC"). Unlike sham-OVX females, whose nociplastic mechanical hypersensitivity was alleviated by local bupivacaine (**Figure 16A**), OVX females resembled males in that afferent silencing at the previously injured area did not attenuate nociplastic mechanical hypersensitivity (**Figure 16B**). This suggested that female gonadal hormones are required to make nociplastic mechanical hypersensitivity dependent upon ongoing afferent input at the previously injured area.

Estrogen reconstitution prior to, but not after, postinjury stimulation is sufficient to induce the peripherally maintained nociplastic pain state

We subsequently considered whether this female gonadal hormone-dependent nociplastic pain mechanism was estrogen-dependent, as estrogen has been implicated in female-specific hyperalgesic priming, one of the pain chronification mechanisms (Araldi et al., 2017; Ferrari et al., 2016). Thus, OVX females were implanted with an osmotic mini pump containing 17β-estradiol (18 μg/mL) prior to capsaicin+40°C, which produced

physiological range serum estradiol levels of 64.6 ± 7.8 pg/mL (n = 4) (Barkley et al., 1979) that corresponded to previous publications (Lee et al., 2018; Moran et al., 2007; Ström et al., 2012; Vacca et al., 2016). After one week of 17β-estradiol supplementation, reconstituted OVX (OVX+E2) females were injected with capsaicin and stimulated with 40°C to induce the transition to a nociplastic pain state. After 7 to 10 days, local anesthesia of the previously capsaicin-injected area immediately and effectively attenuated nociplastic mechanical hypersensitivity in OVX+E2 females (**Figure 17A**), like in gonad-intact females. We next asked whether supplementing 17β-estradiol 'after' triggering the transition in OVX females would also restore the responsiveness of nociplastic mechanical hypersensitivity to such local anesthesia. Accordingly, we reconstituted a subset of OVX females with 17β-estradiol 4 days after capsaicin+40ºC (OVX+delayed E2), after the transition to a nociplastic pain state had presumably occurred (Hankerd et al., 2021). After 6 days of 17β-estradiol supplementation, afferents at the previously capsaicin-injected area were silenced with local bupivacaine. In this experiment, the anesthesia of previously capsaicin-injected area did not alleviate nociplastic mechanical hypersensitivity, such that OVX+delayed E2 females resembled unsupplemented OVX females and males (**Figure 17B**). As estrogen supplementation after pain chronification has been demonstrated to alleviate neuropathic pain (Lee et al., 2018), we further assessed whether continued 17β-estradiol supplementation would similarly reduce the magnitude of nociplastic mechanical hypersensitivity. However, in contrast to neuropathic pain models, we found that prolonged 17β-estradiol reconstitution after the transition to a nociplastic pain state did not alleviate nociplastic mechanical hypersensitivity in OVX+delayed E2 females (**Figure 18**). Together, these results suggested that the presence of 17β-estradiol is sufficient to make

females to develop peripherally maintained nociplastic pain state upon transition triggering events; however, once the transition has occurred in the absence of female gonadal hormones, the maintenance mechanisms of nociplastic pain state is neither switched back to that of gonad-intact females nor is the magnitude of persistent mechanical hypersensitivity reduced by estrogen supplementation.

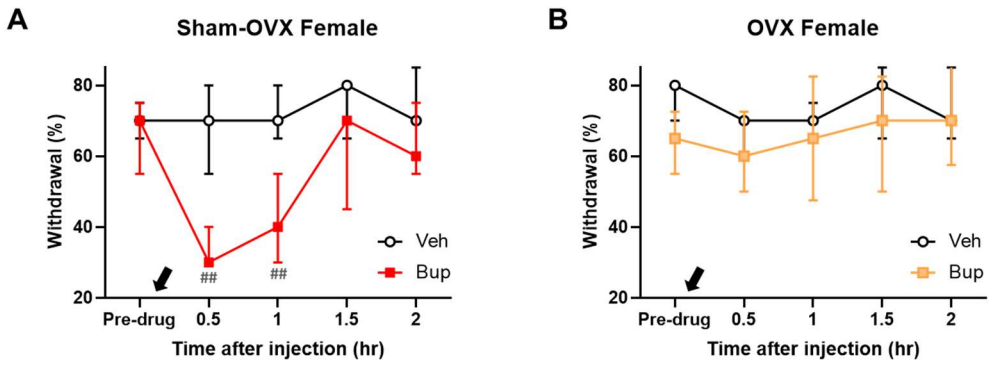

Figure 16: Ovariectomy makes nociplastic mechanical hypersensitivity independent of ongoing peripheral afferent firing

Local injection of bupivacaine (Bup, 0.75%, black arrow) 7 to 10 days after capsaicin+40ºC attenuated nociplastic mechanical hypersensitivity outside of the previously capsaicin-injected area in (**A**) sham-ovariectomized (Sham-OVX) but not (**B**) ovariectomized (OVX) females. ##p<0.01 vs pre-drug level by sequential Sidak test. Data are presented as median with interquartile range. Sham-OVX females: n = 5 per group. OVX females: n = 5 in vehicle (Veh) and n = 6 in Bup.

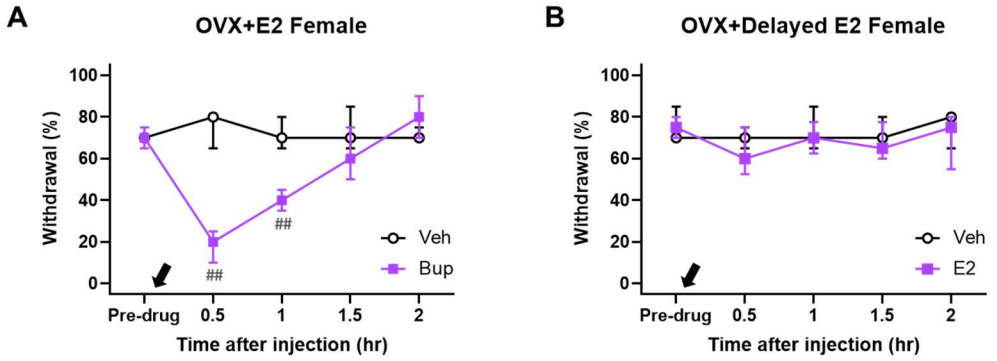

Figure 17: 17β-estradiol supplementation prior to, but not after, postinjury stimulation is sufficient to make nociplastic mechanical hypersensitivity dependent upon ongoing peripheral afferent firing

(**A**) In ovariectomized females supplemented with 17β-estradiol prior to capsaicin+40°C (OVX+E2), silencing peripheral afferents at the previously capsaicin-injected area with bupivacaine (Bup, 0.75%, black arrow) 7 to 10 days after capsaicin+40°C attenuated nociplastic mechanical hypersensitivity outside of the previously capsaicin-injected area. (**B**) When ovariectomized females were reconstituted with 17β-estradiol 4 days after capsaicin+40°C (OVX+delayed E2), local bupivacaine (black arrow) did not attenuate nociplastic mechanical hypersensitivity in OVX females. ##P<0.01 vs pre-drug level by sequential Sidak test. Data are presented as median with interquartile range. OVX+E2 females, n = 5 per group. OVX+delayed E2, n = 5 in vehicle (Veh) and n = 4 in bupivacaine. E2, 17β-estradiol.

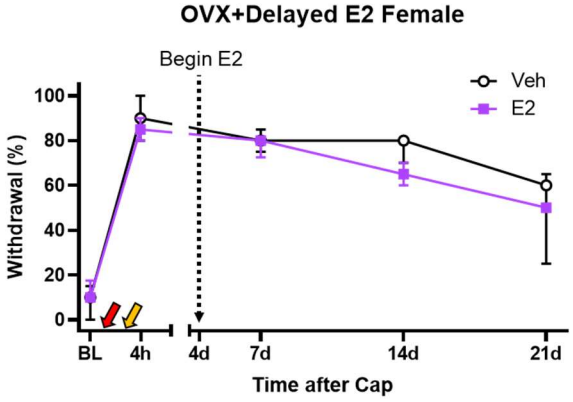

Figure 18: 17β-estradiol supplementation does not reduce the magnitude of nociplastic mechanical hypersensitivity

Supplementing ovariectomized females with 17β-estradiol (E2, dotted line) 4 days after capsaicin (Cap, red arrow) + 40°C (yellow arrow) did not alter the magnitude of nociplastic mechanical hypersensitivity. Data are presented as median with interquartile range. In vehicle (Veh), n = 5. In E2, n = 4.

Estrogen receptor GPER mediates the development and maintenance of the peripherally maintained nociplastic mechanical hypersensitivity in gonad-intact females

The estrogen receptor GPER has been implicated in the augmentation of postoperative pain (J. J. Xu et al., 2021) and hyperalgesic priming in females (Araldi et al., 2017). Thus, we next asked whether GPER mediated the development of the peripherally maintained nociplastic pain state in gonad-intact females. Accordingly, the GPER antagonist G-36 (50 μg/kg) was systemically injected in gonad-intact females for 4 days during the transition to a nociplastic pain state (**Figure 14**, specifically, immediately following 40°C and once daily from days 1 to 3 post-capsaicin). When peripheral afferents at the previously capsaicin-injected area were silenced with local bupivacaine 7 to 10 days after the capsaicin injection, nociplastic mechanical hypersensitivity was not attenuated (**Figure 19A**), suggesting that the activity of estrogen receptor GPER is required for the development of a peripherally maintained nociplastic pain state. We next assessed the contribution of GPER to the maintenance of nociplastic mechanical hypersensitivity in gonad-intact females. Specifically, we asked whether GPER antagonism after the transition to a nociplastic pain state would make the maintenance peripherally independent or, alternatively, trigger the resolution of nociplastic mechanical hypersensitivity. Thus, as a counterpart to our previous experiment, gonad-intact females received daily systemic injections of G-36 4 days after capsaicin+40ºC (i.e., after the transition to a nociplastic pain state had occurred). Gonad-intact females receiving delayed G-36 showed gradual attenuation of mechanical hypersensitivity, as compared to their vehicle receiving counterparts (**Figure 19B**). This suggested that the maintenance of nociplastic mechanical

hypersensitivity in gonad-intact females requires sustained activity of GPER and downstream signaling cascades.

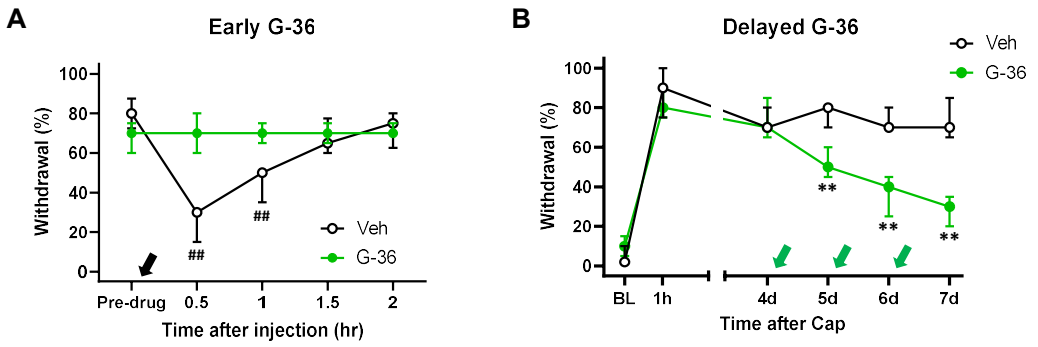

Figure 19: Estrogen receptor GPER activity is critical for both the development and maintenance of the peripherally maintained nociplastic pain state in gonad-intact females

(**A**) Antagonism of the estrogen receptor GPER with G-36 (50 µg/kg) during the transition phase (days 0-4 post-capsaicin+40°C) made nociplastic mechanical hypersensitivity outside of the previously injured area uninhibitable by bupivacaine injection (Bup, 0.75%, black arrow) at the previously capsaicin-injected area 7 to 10 days post-capsaicin. (**B**) After the transition to a nociplastic pain state, daily injections of G-36 (green arrows) gradually reduced the magnitude of nociplastic mechanical hypersensitivity in gonad-intact females. ##P <0.01 vs pre-drug level, **P <0.01 vs vehicle (Veh) by Sidak sequential test. Data are presented as median with interquartile range. Early G-36: n = 4 in Veh and n = 5 in G-36. Delayed G-36: n = 5 per group.

AITC-responsive afferents at the previously capsaicin-injected area maintain nociplastic mechanical hypersensitivity in gonad-intact females

We first identified the afferent population at the previously capsaicin-injected area, which maintains nociplastic mechanical hypersensitivity in gonad-intact females. Co-injection of lidocaine N-ethyl bromide (QX-314), a membrane-impermeable lidocaine derivative, with capsaicin, allyl isothiocyanate (AITC), or flagellin has been demonstrated to selectively silence afferent populations expressing TRPV1 (Puopolo et al., 2013), TRPA1 (Nakagawa

& Hiura, 2013), and Toll-like receptor 5 (TLR5) (Z. Z. Xu et al., 2015), respectively, via inhibition of voltage gated sodium channels (**Figure 20A**). Co-injection of QX-314 with the TRPA1 agonist AITC 7 to 10 days after capsaicin+40ºC (**Figure 20B**: pre-drug vs. post-drug levels at 0.5 hour: $t(49) = 3.19$ by sequential Sidak test, $P=0.01$), but not with capsaicin (**Figure 20C**) or flagellin (**Figure 20D**), alleviated nociplastic mechanical hypersensitivity in gonad-intact females. This suggested that AITC-responsive afferents are persistently active at the previously capsaicin-injected area, maintaining nociplastic mechanical hypersensitivity in gonad-intact females.

AITC-responsive afferents maintain nociplastic mechanical hypersensitivity in gonad-intact females independently of TRPA1 activation

Because AITC is the agonist of TRPA1 (Takaya et al., 2015), these AITC-responsive afferents likely express TRPA1 (Bandell et al., 2004; Jordt et al., 2004). Thus, we next asked whether the ongoing activity of AITC-responsive afferents at the previously capsaicin-injected area was due to endogenous activation of TRPA1. Accordingly, 7 to 10 days post-capsaicin+40°C, TRPA1 at the previously capsaicin-injected area was inhibited by local injection of the TRPA1 antagonist HC-030031 (**Figure 21A**, 30 μM). TRPA1 antagonism failed to attenuate nociplastic mechanical hypersensitivity (**Figure 21B**). This result indicated that the nociplastic pain state in gonad-intact females is maintained by sensitized AITC-responsive afferents does not involve TRPA1 activation.

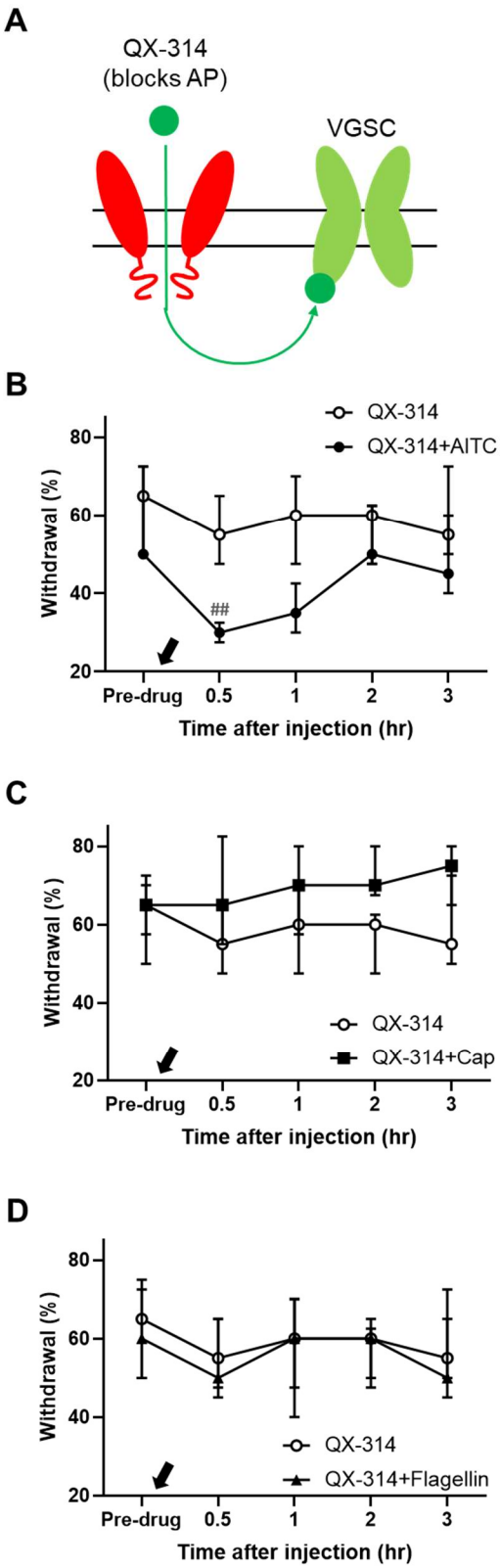
A
QX-314
(blocks AP)
VGSC
B
QX-314
QX-314+AITC
Withdrawal (%)
80
60
40
20
##
Pre-drug
0.5
1
2
3
Time after injection (hr)
C
Withdrawal (%)
QX-314
QX-314+Cap
Time after injection (hr)
D
Withdrawal (%)
QX-314
QX-314+Flagellin
Time after injection (hr)

Figure 20: Selectively silencing AITC-responsive afferents alleviates nociplastic mechanical hypersensitivity in gonad-intact females 7 to 10 days post-capsaicin+40°C

(**A**) QX-314 enters the cell via large pore channels, whereby it binds to voltage-gated sodium channels (VGSC) to prevent specific afferent subpopulations from firing action potentials (AP). Co-injection of QX-314 (2%, black arrow) with (**B**) allyl isothiocyanate (AITC, 30 μM), but not (**C**) capsaicin (Cap, 0.1%) or (**D**) flagellin (0.9 μg), at the previously capsaicin-injected area 7 to 10 days post-capsaicin+40°C significantly alleviated nociplastic mechanical hypersensitivity outside of the previously capsaicin-injected area in gonad-intact females. ##P ≤0.01, pre-drug vs post-drug level by sequential Sidak test. Data are presented as median with interquartile range. Vehicle (Veh, QX-314), QX-314 + AITC, and QX-314 + cap, n = 6; QX-314 + flagellin, n = 5.

Increases in the mean spontaneous firing frequency and decreases in the mechanical threshold of AITC-responsive afferents innervating the capsaicin+40°C area are female gonadal hormone-dependent

Focusing on AITC-responsive afferents, which are small to medium diameter mechanosensitive primary afferent neurons (Barabas et al., 2012), we determined if these AITC-responsive afferents innervating the previously capsaicin-injected area show ongoing activity after capsaicin+40°C. Employing single-unit extracellular recordings in *ex vivo* skin-nerve preparations (ipsilateral to the capsaicin injection) obtained 7 to 10 days after capsaicin alone or capsaicin+40°C, we measured properties of afferents innervating the previously capsaicin-injected area. Recordings from the contralateral hind paw and naïve mice were pooled as controls. Afferents that fired burst action potentials or whose baseline firing rate doubled when AITC (30 μM) was applied topically at the end of each recording were regarded as 'AITC-responsive'.

We first examined changes in spontaneous firing. In our experimental configuration, 60% of afferents (342 of 563 units from 83 animals) showed spontaneous firing at various frequencies (0.001-2.5 Hz). While there was no difference in the percentage of spontaneously active afferents between groups in gonad-intact females and males regardless of AITC-responsiveness, in OVX females, a significantly greater proportion of AITC-responsive units was spontaneously active in capsaicin+40˚C group (75.0%) than in control (45.0%) ($t(199) = 2.487$, $P=0.041$). However, this proportional increase did not lead to an increase in their mean spontaneous firing frequency in OVX females (see **Figure 22D**). Next, focusing on the spontaneously active units, we assessed whether AITC-responsive afferents in capsaicin+40°C group fired at higher frequency than those in control or capsaicin alone group. In gonad-intact females, we found a significant increase in the mean spontaneous firing frequency of AITC-responsive afferents in capsaicin+40°C group as compared to their counterparts in capsaicin alone group (**Figure 22A**: $t(113) = 2.543$, $P=0.037$) and a robust trend toward increase as compared to AITC-responsive afferents in control group ($t(113) = 2.234$, $P=0.054$). Notably, in gonad-intact females, such increase in mean spontaneous firing frequency was not observed in AITC-unresponsive units in capsaicin+40°C group. Furthermore, the mean spontaneous firing frequency of AITC-responsive units did not differ between groups in OVX females (**Figure 22D**) or males (**Figure 22G**).

Because AITC is the agonist of TRPA1, which confers mechanosensitivity to nociceptors (Kwan et al., 2009; Vilceanu & Stucky, 2010), we additionally assessed whether the mechanosensitive properties of AITC-responsive afferents from gonad-intact females were altered by capsaicin+40°C. We found a significant decrease in the

mechanical threshold of AITC-responsive afferents in capsaicin+40ºC group from gonad-intact females (**Figure 22B**), but not OVX females (**Figure 22E**) or males (**Figure 22H**). Specifically, in gonad-intact females, the mechanical threshold of AITC-responsive units in capsaicin+40ºC group was significantly decreased compared to their counterparts in capsaicin alone group (**Figure 22B**: t(108) = -3.693, P=0.001); there was also a trend toward decrease compared to AITC-responsive units in control group (**Figure 22B**: t(108) = -1.743, P=0.084). However, there were no differences in the total number of action potentials evoked by ramp mechanical stimulation (0-200 mN) from AITC-responsive afferents between groups regardless of the sex hormonal state (i.e., gonad-intact females (**Figure 22C**), OVX females (**Figure 22F**), or males (**Figure 22I**)).

Collectively, this data suggested that capsaicin+40°C renders AITC-responsive afferents innervating the capsaicin-injected area spontaneously active at a high-frequency and decreases the threshold only in gonad-intact females.

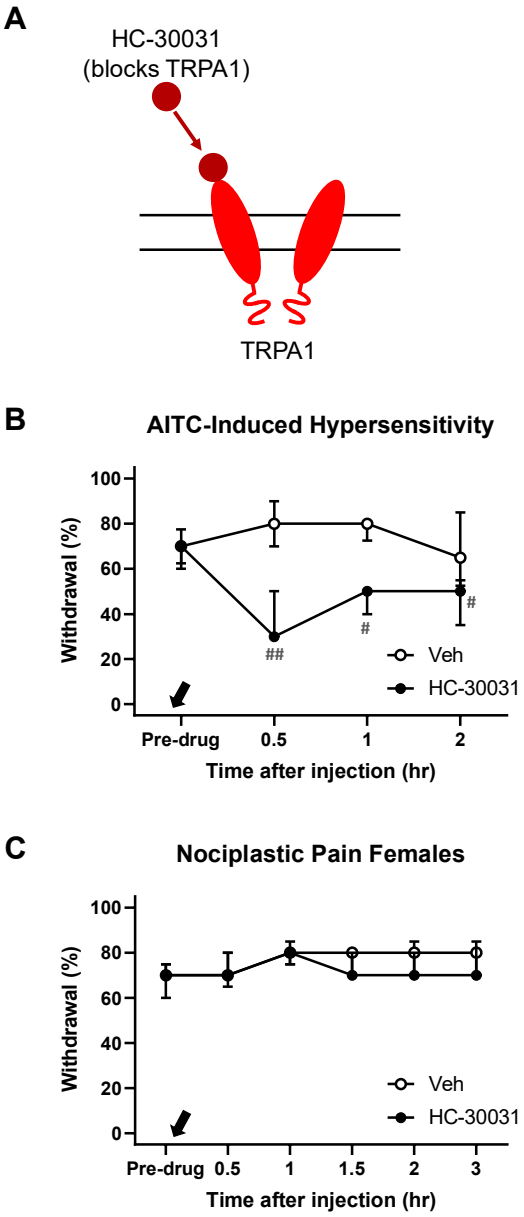

Figure 21: TRPA1 antagonism 7 to 10 days post-capsaicin+40°C does not attenuate persistent mechanical hypersensitivity in gonad-intact females

(**A**) Unlike QX-314, which prevents specific afferent populations from firing action potentials by inhibiting voltage-gated sodium channels, HC-30031 binds directly to and blocks the activity of Transient Receptor Potential A1 (TRPA1). (**B**) HC-030031 (30 µM, black arrow) 0.5 hr after intradermal injection of allylisothiocyanate (AITC, 0.1%) attenuates AITC-induced mechanical hypersensitivity. (**C**) Antagonizing TRPA1 by local injection of HC-30031 (30 µM) at the previously capsaicin-injected area did not attenuate nociplastic mechanical hypersensitivity outside of the area in gonad-intact females. Data are presented as median with interquartile range. AITC model: n = 4 in vehicle (Veh)

and n = 5 in HC-030031. Nociplastic pain females: n = 5 per group.

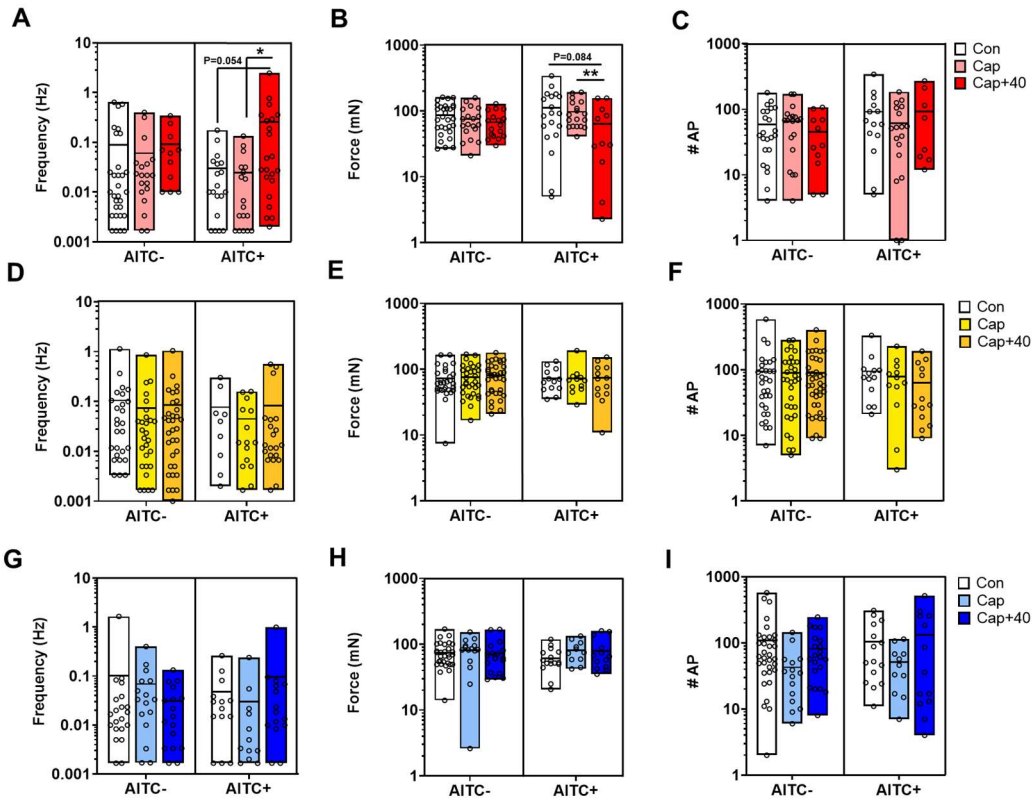

Figure 22: AITC-responsive afferents have an increased mean spontaneous firing frequency and a decreased mechanical threshold in a female gonadal hormone-dependent manner 7-10 days post capsaicin+40ºC

There were no significant differences between groups (control, Con; capsaicin, Cap; capsaicin+40ºC, Cap+40) in the mean spontaneous firing frequency of AITC-nonresponsive ($AITC^-$) units. However, in (**A**) gonad-intact females, there was a significant increase in the mean spontaneous firing frequency of $AITC^+$ units in Cap+40, as compared to Cap, and a trending increase versus Con. This was not observed in (**D**) ovariectomized females or (**G**) males, as compared to $AITC^+$ units in Con or Cap. *P <0.05 for Cap+40 vs. Cap by sequential Sidak test. The bottom, middle line, and top of each bar indicate the minimum, mean, and maximum values in each group, respectively. Gonad-intact females: $AITC^-$ units: n = 30 in Con, in Cap n = 19, and n = 11 in Cap+40; $AITC^+$ units: n = 19 in Con, n = 18 in Cap, n = 22 in Cap+40. Ovariectomized females: $AITC^-$ units: n = 27 in Con, n = 28 in Cap, and n = 30 in Cap+40; $AITC^+$ units, n = 9 in Con, n =15 in Cap, and n = 22 in Cap+40. Males: $AITC^-$ units: n = 21 in Con, n = 16 in Cap, n = 17 in Cap+40; $AITC^+$ units: n = 14 in Con, n = 12 in Cap, n = 15 in Cap+40. In (**B**) gonad-intact females, but not (**E**) ovariectomized females or (**H**) males, the mechanical

threshold was significantly decreased as compared to Cap counterparts and there was a trending decrease versus Con. There was no significant differences in the total number of ramp-evoked action potentials (AP) from capsaicin+40ºC (**C**) gonad-intact females, (**F**) ovariectomized females, or (**I**) males. **P <0.01 for Cap+40 vs. Cap by sequential Sidak test. For statistical analysis, the threshold values were log transformed. The bottom, middle line, and top of each bar indicate the minimum, mean, and maximum values in each group, respectively. Gonad-intact females: $AITC^-$ units: n = 30 in Con threshold and n = 32 in Con #AP, n = 19 in Cap, and n = 17 in Cap+40; $AITC^+$ units: n = 19 in Con, n = 19 in Cap, n = 13 in Cap+40 threshold and n = 11 in Cap+40 #AP. Ovariectomized females: $AITC^-$ units: n = 30 in Con, n = 32 in Cap, and n = 34 in Cap+40; $AITC^+$ units, n = 13 in Con, n =12 in Cap, and n = 13 in Cap+40. Males: $AITC^-$ units: n = 31 in Con, n = 15 in Cap, n = 19 in Cap+40; $AITC^+$ units: n = 16 in Con, n = 11 in Cap, n = 13 in Cap+40.

DISCUSSION

In this study, we found that the transition from acute injury-induced pain to nociplastic pain still occurred in female mice in the absence of female gonadal hormones (i.e., after OVX), which corresponds to clinical reports that the incidence of CRPS type I, an exemplary chronic pain condition presenting with nociplastic pain, persists beyond menopause (≥ 60-year-old women) (Sandroni et al., 2003). Of note, the absence of female gonadal hormones changed the behavioral phenotype of females to that of males in that their nociplastic mechanical hypersensitivity was unaffected by the local anesthesia of previously injured area. Because supplementing only estrogen after OVX was sufficient to restore the behavioral phenotype of gonad-intact females and inhibiting the estrogen receptor GPER was sufficient to mimic the effect of OVX in gonad-intact females, estrogen, acting through GPER, must be the key female sex hormone that makes the nociplastic pain state maintained by ongoing afferent activity at the previously injured area in females. Regarding this notion, however, it should be clarified that supplementing OVX females

with estrogen 4 days "after" capsaicin+40ºC failed to make either the nociplastic mechanical hypersensitivity sensitive to bupivacaine injection at the previously injured area or the hypersensitivity attenuated over time. This suggests that, once the transition has occurred in the absence of estrogen, the mechanism(s) of nociplastic pain is irreversibly set to being independent of estrogen and ongoing afferent activity at the previously injured area. Thus, it would be interesting to ascertain whether the mechanisms driving nociplastic pain in postmenopausal women are unaltered by estrogen-based hormone replacement therapy, which is prescribed to alleviate menopausal symptoms.

Our behavioral and electrophysiological results implicate AITC-responsive afferents (presumably TRPA1-expressing nociceptors) as the culprit that peripherally maintains the nociplastic pain state in gonad-intact females. Specifically, co-administration of AITC and QX-314 at the previously injured area significantly inhibited nociplastic mechanical hypersensitivity, suggesting that AITC-responsive afferents at the previously injured area are spontaneously and persistently active. Supporting this notion, we found that the mean spontaneous firing frequency of AITC-responsive afferents was increased and the mechanical threshold decreased only in the gonad-intact female nociplastic pain model. Though the mechanisms driving these AITC-responsive afferents to become spontaneously and persistently active are unknown, it seems unlikely that this spontaneous firing requires "endogenous opening" of large pore channels like TRPA1, because TRPA1 antagonism and local injection of QX-314 alone was unable to inhibit the nociplastic mechanical hypersensitivity. Previously we identified selective increases in the gene expression of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) 1 day after capsaicin+40ºC at the capsaicin-injected area only in the female nociplastic pain model.

As these two cytokines are known to induce hyperalgesic priming in nociceptors, which also drives pain chronification (Dina et al., 2008; Parada et al., 2003), hyperalgesic priming-like mechanisms may render AITC-responsive afferents spontaneously and persistently active. In this regard, it is noteworthy that ~80% of TRPA1-expressing dorsal root ganglion neurons are isolectin B4 (IB4)-binding in rodents (Barabas et al., 2012), and IB4-binding afferents are required for hyperalgesic priming (Joseph & Levine, 2010).

Interestingly, after recovering from an initial priming injury (e.g., intraplantar IL-6 or TNF-α injection), animals show normal mechanical sensitivity unless challenged by a second injury. In our female nociplastic pain model, the increased gene expression of IL-6 or TNF-α is no longer present at 7 days post-capsaicin. It should be clarified here that intraplantar injection of IL-6 or TNF-α produces mechanical hypersensitivity *per se* (Avona, Price, et al., 2021; Woolf et al., 1997) and inflammation increases mechanosensitivity of AITC-responsive afferents (Dunham et al., 2008; Lennertz et al., 2012). Therefore, it could be that AITC-responsive afferents develop substantial mechanical hypersensitivity together with spontaneous activity early on after capsaicin+40°C, and the spontaneous activity and decreased mechanical threshold persists even after the initial inflammation has resolved.

Although the exact mechanism(s) of the persistent, high frequency spontaneous activity and decreased threshold of AITC-responsive afferents are currently unknown, this study clearly indicates that the AITC-responsive afferents require female gonadal hormones to develop such ongoing activity. Estrogen, which was found to be the key sex hormone for the development of peripherally maintained nociplastic pain state in this study, is well known to mediate transcriptional-translational changes through GPER, as reported

in hyperalgesic priming (Araldi et al., 2017). As GPER activation has been demonstrated to activate mTORC1 (Bian et al., 2019; Wang et al., 2020), PKCε (Kuhn et al., 2008), and ERK (Filardo et al., 2000; Liverman et al., 2009), future studies will elucidate signaling cascades responsible for AITC-responsive afferent sensitization.

There are multiple questions remain to be answered. First, it is unclear how the nociplastic pain state in OVX females is maintained, as their nociplastic mechanical hypersensitivity was unaffected by the local anesthesia of previously injured area, like male's. In our previous study, activated spinal microglia maintain the nociplastic pain state in males. Does this suggest that OVX females might similarly utilize a microglia-dependent mechanism to maintain their nociplastic pain state? Female gonadal hormones modulate the immune system (Klein & Flanagan, 2016; Taneja, 2018), including glial activation (Lee et al., 2018; Wu et al., 2016), and clinical data suggests that sex differences in the adaptive immune system (Giefing-Kröll et al., 2015) diminish after menopause. However, preclinical studies suggest that spinal microglia activation in neuropathic pain states is either testosterone-dependent or genetically encoded (Mapplebeck et al., 2018; Sorge et al., 2015). Thus, the removal of female gonadal hormones alone might not be sufficient to make females develop a microglia-driven nociplastic pain state. As it is clinically relevant to chronic pain in postmenopausal women, the exact nature of the nociplastic pain mechanisms in OVX females is our immediate research interest in a future study. Second, although here we identified GPER as the key estrogen receptor mediating the peripherally maintained nociplastic pain state in a gonad-intact females, we recognize that the contribution of estrogen receptors α and β to nociplastic mechanical hypersensitivity must be explored for a comprehensive understanding of the sex hormone's role in nociplastic

pain. Lastly, we acknowledge that the contribution of additional afferent populations to the maintenance of female nociplastic pain state is unknown because the silencing of specific afferent populations is constrained to capsaicin-sensitive, AITC-sensitive, and flagellin-sensitive afferents in this study. Thus, any remaining contribution by other afferent populations that cannot be silenced by this approach to nociplastic pain maintenance in gonad-intact females needs to be addressed.

In conclusion, we have found that high-frequency spontaneous firing and decreased mechanical threshold of AITC-responsive afferents innervating the capsaicin+40°C area occurs only in gonad-intact females. These findings support the role of female sex hormones in nociplastic pain mechanisms rather than its development and call for further investigation into the gonadal hormone-independent mechanism of nociplastic pain in females, which is relevant to postmenopausal women suffering from nociplastic pain conditions.

Chapter 5: General conclusions and future directions

Though injury-induced pain normally subsides as the injury and local inflammation resolves, in some patients this nociceptive pain transitions into nociplastic pain. Replicating these patients, we developed a murine model in which normally resolving injury-induced pain transitions into a nociplastic pain state in the absence of overt tissue or somatosensory damage or inflammation (Hankerd et al., 2021). Notably, though both sexes develop nociplastic mechanical hypersensitivity, female mice were more susceptible to developing a nociplastic pain state, which could account for the female predominance of nociplastic pain conditions. We additionally identified sexually dimorphic mechanisms maintaining the nociplastic pain state. As women are predominately diagnosed with nociplastic pain conditions (Melchior et al., 2016; Sandroni et al., 2003), the studies presented here have focused on the female-specific mechanisms. We found that peripherally maintained nociplastic mechanical hypersensitivity was female gonadal hormone-dependent and that, in gonad-intact females with nociplastic pain, the pain state was maintained by ongoing activity of AITC-responsive afferents at the previously injured area.

It is noteworthy that the mean spontaneous firing frequency of AITC-responsive afferents firing spontaneously at a high frequency was not increased and mechanical threshold decreased in males and OVX females with nociplastic pain. This suggests that AITC-responsive afferent sensitization by postinjury stimulation requires female gonadal hormones. While the exact mechanisms of AITC-responsive afferent sensitization in gonad-intact females remains unknown, it is likely that signaling cascades downstream of estrogen (likely acting through GPER) contribute to AITC-responsive afferent sensitization. As mechanical hypersensitivity was measured 24 hours after each G-36

administration, this effect is likely due to GPER-mediated long-term transcriptional-translational changes (Bian et al., 2019; Filardo et al., 2000; Hyder et al., 1991; Kuhn et al., 2008; Liverman et al., 2009), rather than rapid antinociceptive actions of GPER (An et al., 2014; Lu et al., 2013). Thus, future studies will identify the GPER-mediated transcriptional-translational changes contributing to the development and maintenance of the peripherally maintained nociplastic pain state in gonad-intact females. Of particular interest are transcripts expressed in AITC-responsive afferents that contribute to increased spontaneous firing, such as voltage-gated sodium channels (Bennett et al., 2019), and transcripts involved in pain chronification, such as estrogen-dependent prolactin upregulation (Patil et al., 2019), which promotes the pain chronification in the form of hyperalgesic priming (Paige et al., 2020).

As discussed in the previous chapter, the mechanisms governing the development and maintenance of nociplastic pain in OVX females are unclear. Our preliminary behavioral studies suggest that spinal microglia do not maintain nociplastic mechanical hypersensitivity in OVX females, as microglial inhibition by Mac-1-saporin 7 to 10 days post-capsaicin+40°C did not alleviate nociplastic mechanical hypersensitivity. Studies on microglia activation in neuropathic pain conditions indicate that microglial activation may be male-specific and may require the presence of testosterone (Sorge et al., 2015), which is low in OVX females (Nilsson et al., 2015). Alternatively, microglial activation in males, but not gonad-intact or OVX females, could result from sex-specific receptor profiles and reactivity (Mapplebeck et al., 2018; Sorge et al., 2015), and differential microglial maturation by peri- and post-natal hormone exposure (Acosta-Martínez, 2020; Han et al., 2021). Thus, studies are warranted to identify the mechanisms maintaining nociplastic

mechanical hypersensitivity in OVX females (which is relevant to postmenopausal women) and to identify the factor(s) mediating spinal microglial activation by postinjury stimulation.

In conclusion, we have developed a murine model that allows for mechanistic studies on both transition and maintenance mechanisms of nociplastic pain. Importantly, we have identified biological female susceptibility to developing nociplastic pain and pinpointed female gonadal hormones as a critical mechanistic driver of the peripherally maintained nociplastic pain state, whereby AITC-responsive afferents innervating the previously injured area fire spontaneously at a high frequency and have a decreased mechanical threshold in a female gonadal hormone-dependent manner (**Figure 23**). We expect that these findings will promote the development of therapeutics to prevent and treat nociplastic pain with sex- and/or hormonal stage-specific approaches.

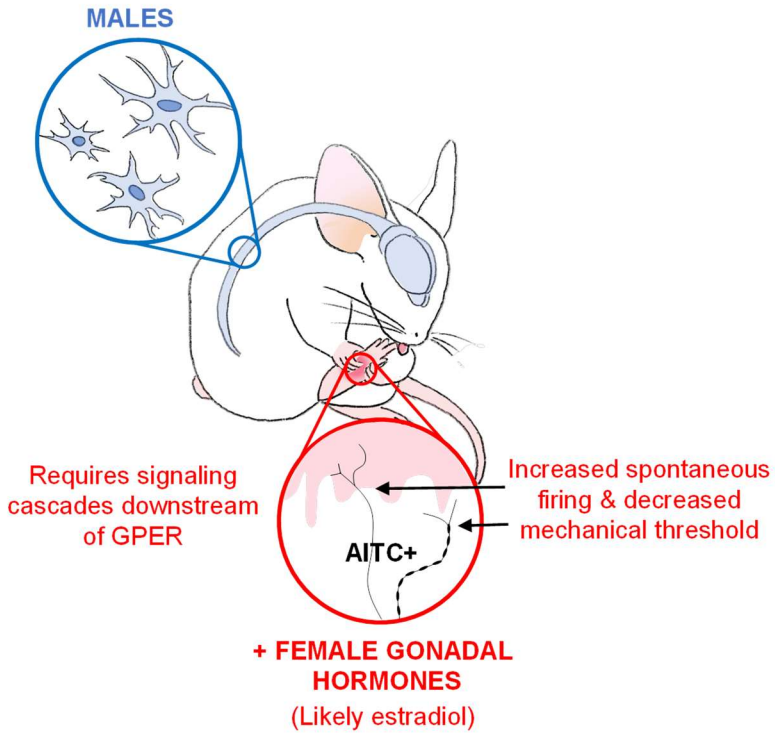

Figure 23: Proposed maintenance mechanisms of nociplastic mechanical hypersensitivity

While both sexes develop nociplastic mechanical hypersensitivity in the absence of overt ongoing local inflammation at the previously injured (i.e., capsaicin-injected) area, the maintenance mechanisms are sexually dimorphic. In gonad-intact females, the presence of female gonadal hormones (especially estrogen) renders AITC-responsive ($AITC^+$) afferents at the previously injured area increases spontaneous firing and decreases mechanical threshold. AITC-responsive afferent sensitization is maintained by signaling cascades downstream of the G protein-coupled estrogen receptor (GPER). In males, activated spinal microglia maintain nociplastic mechanical hypersensitivity. Figure drawn by Kali Hankerd.

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Publications

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