Rostral ventromedial medulla (RVM) pain modulating neurons in male and female rats with persistent pain

By

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CONTENTS

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LIST OF ABBREVIATIONS

ACC	Anterior cingulate cortex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CeA	Central nucleus of the amygdala
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
EKG	Electrocardiograph
EMG	Electromyograph
GABA	γ-Aminobutyric acid
IBS	Irritable bowel syndrome
Lat.	Latency
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartate
PAG	Periaqueductal gray
PB	Parabrachial nucleus
QST	Quantitative sensory testing
RVM	Rostral ventromedial medulla

SNL	Spinal nerve ligation
SP	Substance P
vF	von Frey fibers

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ABSTRACT

The importance of understanding sex differences in patients with clinical pain has spurred investigations of real and potential sex differences in pain transmission and modulation. In pre-clinical and rodent models, differences in basal pain thresholds or sensitivity have been identified with multiple stimuli (Mogil et al., 1997) but these results are often inconsistent (Grisel & Mogil 2000), and frequently confounded by test conditions (Hashmi & Davis 2014, Mogil et al., 1998). Although female and male rodents commonly display similar pain thresholds, observed differences in the development, response to and resolution of persistent pain suggest that sex differences may arise in endogenous pain modulation. The development of chronic pain is increasingly recognized as a shift to a pathological state of endogenous pain modulation (Yarnitsky 2015), defined by a number of different short- and long-term modifications in the central nervous system that increase or decrease pain (Woolf 2011). Over the years, research into endogenous pain modulation has identified a critical central circuit which acts via the rostral ventromedial medulla (RVM), the output node of this descending pain-modulating circuitry. This brainstem area can both facilitate and inhibit nociceptive transmission by the action of two neuron classes: ON cells, which elicit a burst of action potentials in response to a noxious stimulus, and OFF-cells, which respond with a pause in their ongoing firing. Changes within the RVM contribute to the behavioral hypersensitivity that develops during the transition to chronic pain. However, whether there is sexual dimorphism in the physiology of these RVM pain-modulating neurons is unknown.

To addresses this gap in our knowledge, I used single-neuron extracellular recording and pain testing in a cohort of 52 male and 54 female rats to determine whether the physiological responses of pain-modulating neurons of the RVM differ between the sexes and whether this difference is altered by the induction of persistent

inflammatory pain. Naive males and females had similar thermal and mechanical withdrawal thresholds, although the latency to withdrawal from the mechanical stimulus was shorter for males. Nevertheless, RVM neuronal activity was similar between the sexes under basal conditions. Additionally, after induction of persistent inflammation, males and females exhibited similar mechanical hypersensitivity, and showed no significant differences in responses of RVM neurons.

Collectively, these data indicate that although there are activational differences in other supraspinal regions related to pain transmission, including the parabrachial nucleus and periaqueductal gray which provide critical efferent inputs to pain modulating neurons, I did not detect sexual dimorphism in RVM responses.

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

The complexity of the clinical response to pain has confounded the search for the biological substrate of sex differences in chronic pain. However, sex differences in the prevalence of certain chronic pain conditions (Arout et al., 2018, Kim & Kim 2018, Pavlovic et al., 2017, Plesh et al., 2011) have sparked interest into whether there is sexual dimorphism in the mechanisms of pain modulation. Although the rostral ventromedial medulla (RVM)—the output node of the descending neural circuits involved in pain transmission and modulation—has been extensively studied in male rodents, whether the physiology of the RVM differs between males and females is unknown. Additionally, although changes in the neuronal activity within the RVM contribute to behavioral hypersensitivity after induction of persistent inflammation, whether these changes follow a similar pattern in females has not been assessed. The goal of this work was to characterize the activity of pain-modulating neurons in naive female rodents and females in a persistent pain state, and compare their behavioral sensitivity and associated cell activity to males. My dissertation is divided into three chapters: 1, an introduction to provide an overview of the literature context for the work; 2, a manuscript collecting the experiments performed and their results; 3, an overall discussion of the work performed.

Chapter 1 provides a brief overview of the clinical and basic research literature describing differences in the prevalence and development of chronic pain symptoms between men and women. I then review studies evaluating whether there are sex differences in basal sensitivity, and some social, psychological, and experimental variables that influence the detection of sex differences in pain threshold and tolerance. Next, I discuss the anatomy and function of the descending pain-modulating circuitry, as well as activational and pharmacological differences between males and females that

have been identified within this circuitry that could contribute to sex differences in the experience of pain.

In Chapter 2, I describe experiments designed to assess whether there are basal differences in the physiology of pain-modulating neurons of the RVM in male and female rats, as well as whether there are differences in behavioral sensitivity to noxious thermal and mechanical stimuli. Although single-unit recording of RVM neurons has been performed in males for over three decades, in that time only two studies have evaluated RVM neurons in females. Neither tested females in a persistent pain state. My final experiment examined persistent-inflammation-induced changes in RVM activity in females, and compared then to changes seen in males.

In Chapter 3, I discuss my findings within the field of pain research. In summary, despite sexual dimorphism at other relays in the circuits that transmit and modulate pain information, at the level of the RVM males and females appear to be quite similar physiologically, and this similarity is maintained even after the induction of persistent inflammatory pain. Overall, the work contributes to a better understanding of the neural mechanism of pain-transmission and modulation in female subjects and proposes an approach to the study of the mechanisms of pain transmission and modulation.

1.2. SEX DIFFERENCES IN THE PREVALENCE OF CLINICAL PAIN

Most pain patients are women, but we do not know why. As a consequence of this, there is a great deal of interest in understanding what potential mechanisms drive sex differences in clinical pain experiences and their neurobiological substrates. However, the mechanisms underlying the increased prevalence of pain in women remains elusive.

One suggested reason for women's greater burden of chronic pain is that women are more likely to be diagnosed with a pain condition that is not the result of a traumatic injury. Women are more likely to develop chronic pain than men, and many conditions, such as migraine (Pavlovic et al., 2017); fibromyalgia (Arout et al., 2018); irritable bowel syndrome (IBS) (Kim & Kim 2018); and temporomandibular joint disorder (TMJ) (Plesh et al., 2011) are more likely to have female patient majorities. The relative excess of female patients is even more pronounced for certain conditions such as migraine and fibromyalgia, in which there are as many as 3-4 female patients for every male diagnosed (Arout et al., 2018). Nonetheless, there is evidence that the proportion of female to male patients diagnosed with these conditions has been influenced by diagnostic criteria (Vincent et al., 2013). In the case of fibromyalgia, the use of a pressure pain test is integral to diagnosis, but laboratory-induced pressure pain consistently reveals greater sensitivity in women than men even for healthy subjects (Bartley & Fillingim 2013), potentially excluding many male sufferers.

Other reasons for the greater prevalence of female pain patients may include factors like the significant global disease burden that women bear (Ferrari et al., 2013, Ginsburg et al., 2017, Torre et al., 2017), as well as the fact that women are more likely to seek out medical care across their lifetimes (Bertakis 2009). Although the greater utilization of healthcare by women is partially attributable to routine gynecological and

obstetric care (Sina 2017), chronic pelvic pain remains common among women (2.1-24% of women globally) and poorly managed (Leow et al., 2018). Still, as women seek out care for other reasons, pain complaints are more likely to be found and potentially treated during these contacts with the healthcare system. Indeed, women who receive medical care for any reason are more likely than men to report pain as one of their symptoms (Ruau et al., 2012). In fact, a study of 11,000 patients (Ruau et al., 2012) assessed pain ratings that had been collected during clinical office visits and found that not only were women more likely to report that they were currently experiencing pain, but they chose higher pain ratings than men who also reported feeling pain. In another survey that focused on 10 anatomical regions, women were again more likely than men to report feeling pain in more areas of their bodies (Gerdle et al., 2008). Even when gender-specific conditions such as pelvic pain are excluded from consideration there are still more female than male pain patients (King et al., 2009). So, while social factors related to medical care access certainly influence sex differences in the patient population, there remains general agreement that more women experience pain than men.

1.3. SEX DIFFERENCES IN CLINICAL PAIN INTENSITY

Whether the greater prevalence of chronic pain in women translates to greater pain intensity is unclear. The current accepted definition of pain provided by the International Association for the Study of Pain (IASP) is “An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Vervest & Schimmel 1988).” Within this definition the subjective experience of pain is emphasized; the suffering and disability associated with chronic pain can manifest in changes to cognition and motivation, as well as sensation. Still, pain intensity remains an important clinical measure of the pain experience. The most

common way to assess pain intensity is through self-reports using visual analog or graphical scales that range from no pain to the worst possible pain. Clinical pain ratings influence treatment plans and provide a baseline to assess the success of pain management. Additionally, intense pain (even of short duration) is associated with significant suffering and disability (Doualla et al., 2019, Garbi et al., 2014). However, assessments of the intensity of pain that male and female patients report have yielded inconsistent outcomes. Whether higher pain intensities are reported by female patients (Solheim et al., 2017); by male patients (Barnabe et al., 2012, Fillingim 2003, Keefe et al., 2000); or by neither (Edwards et al., 2003, Robinson et al., 1998, Turk & Okifuji 1999) appears to be dependent upon which clinical pain population is surveyed. This leaves no clear imperative for the clinical response to pain intensity with regard to sex. This does not erase the importance or predictive power of pain severity scores. Indeed, post-surgical pain is associated with the likelihood that an acute injury-related pain becomes persistent (Althaus et al., 2014). However, the results of surveys assessing whether men or women experience greater post-surgical pain intensity are not conclusive. In a retrospective study of clinical pain scores after non-ambulatory surgery, Tighe et al., (Tighe et al., 2014) found that women rated their average pain as more intense than men and were more likely to experience "severe pain events" in which patients rate their pain as at least a 7 on a 0-10 scale. This result was corroborated by a later study, which found that women had slightly higher postoperative pain ratings at all times during the first 24 hours after surgery (Tighe et al., 2015). Higher baseline post-operative pain ratings for women have been reported by a number of other studies (Aubrun et al., 2005, Rosseland & Stubhaug 2004, Taenzer et al., 2000), but the age of the patient may significantly influence this sex difference in pain scores as sex differences are more likely to be reported as the age of patients increases (Zheng et al., 2017). Other studies have also failed to replicate the detection of sex differences in post-operative pain (Frot

et al., 2004) making interpretation of sex differences in post-surgical pain more of a challenge.

Although enhanced sensitivity has often been shown to be a clinically important difference between pain patients and pain-free controls, it is only one of many factors that characterize a chronic pain state. The experience of a pain lasting for at least three months, regardless of intensity, is a defining factor for the diagnosis of chronic pain. Enduring pain is also more likely to be reported by women. In fact, much of what differentiates reports of pain in men and women relates to the functional pain complaints, and differences in pain onset, resolution, and/or transition to persistent pain. Women also seem to have more experiences of widespread pain: when surveyed about pain in 10 anatomical regions, a greater proportion of women report pain in more sites than men (Gerdle et al., 2008). Complex regional pain syndrome, defined as a chronic pain condition that most often affects one limb after an injury (Harden et al., 1999) but often with unknown etiology, is not only more common in women (de Mos et al., 2007) but often spreads to more locations on the body in affected women (Dominguez et al., 2009). Although social and diagnostic factors are certain to play a role in the sex differences the appear in clinical pain, this evidence suggests that key disparities could arise from sexual dimorphism in pain transmission or modulation.

1.4. THEORETICAL FRAMEWORKS IN THE STUDY OF SEX DIFFERENCES IN CLINICAL PAIN

Two main hypotheses have guided much of the investigation into sex differences in chronic pain prevalence, and the potential underlying biological substrates. Based on studies with healthy controls, women have apparently greater pain sensitivity than men for many types of noxious stimuli. One hypothesis is that differences in basal sensitivity to pain, and potentially differences in the mechanisms that establish an individual's basal sensitivity to pain, are the cause of sex differences in clinical pain. The second

hypothesis originates from observations of pain patients who show defects in their ability to modulate pain. Endogenous pain modulation is the process by which nociceptive information is enhanced or suppressed according to the immediate needs of an individual to respond to internal cues and states, or to cues from the environment. Because women are more likely to be diagnosed with chronic pain (which has been connected to defects in pain modulation) it is hypothesized that women may have weaker endogenous pain modulation, or that interactions between female sex and the mechanisms of endogenous pain modulation make women more vulnerable to pathological pain modulation. A review of the literature related to both hypotheses follows.

1.5 SEX DIFFERENCES IN BASAL NOCICEPTIVE THRESHOLDS IN HUMAN SUBJECTS

The need to understand sex differences in chronic pain has led to investigations into whether these differences result from basal pain thresholds or perceptual differences between men and women. Quantitative sensory testing (QST)of healthy men and women overall suggests that women may have lower basal pain thresholds for many somatic stimuli, but especially pressure pain (Barke et al., 2012, Brennum et al., 1989, Chesterton et al., 2003, Fillingim et al., 1999, Fillingim & Ness 2000, Maquet et al., 2004) and electrical stimulation (Lautenbacher & Rollman 1993, Riley et al., , Walker 1998). These differences in basal pain threshold are not due to sensory threshold differences (Fillingim et al., 1998). Although the detection limits for tactile stimuli have occasionally been reported to be lower for women (Boles & Givens 2011) other studies have not replicated this finding (Fillingim et al., 1998). Detection limits are also independent of differences in sensitivity to noxious stimuli (Rolke et al., 2006). Variability in the direction and magnitude of sex differences is nonetheless highly dependent upon the choice of noxious stimulus, the area of the body where the pain testing was

performed, and psychosocial factors. That some studies positively report differences in sensitivity, and others do not, has led some researchers to conclude that sex differences in pain thresholds may be real, but are of limited practical value. As a counterpoint, Riley et al., (1998) performed a meta-analysis assessing measures of pain threshold and tolerance and determined that in many cases, failure to reject the null hypothesis was the result of underpowered studies, and not the absence of an effect of sex.

Despite being a more controlled environment than the clinic, however, situational variables within the research setting can strongly influence whether a sex difference is detected. In many cases this is mediated by reliance on self-reported measures. For example, Levine & De Simone (1991) found that men rated their pain as less intense when they were reporting to a female experimenter, whereas women reported similar pain ratings regardless of experimenter gender. Some studies have attempted to control for gender role and expression biases in measures of pain sensitivity by using quantitative and reflex-based sensory testing. Assessments, such as QST (Reitz et al., 2016), control different properties of the stimulus and testing environment to permit greater precision in measuring function and dysfunction. By controlling the intensity and temporal pattern of an applied thermal stimulus, for example, qualitative descriptions of the evoked pain can be more easily compared across or within subjects. In other cases, the latency before an individual pulls away from a painful stimulus, or the magnitude of muscular contraction as they do so, can stand in for subjective ratings of the pain sensation. However, the use of quantitative testing does not eliminate opportunities for the introduction of bias due to social factors. Mattos Feijo et al., (2018) pointed to the risk of bias in pain study recruiting practices—men who more strongly identified with masculine gender role, and consequently with role-based ideals of pain tolerance, were more likely to volunteer for and participate in pain studies of healthy subjects. As this was not true for women, social issues that skew the experimental population toward an

overrepresentation of men with high pain tolerance could obscure the existence of basal sex differences in pain sensitivity.

1.6 SEX DIFFERENCES IN NOCICEPTIVE THRESHOLDS IN RODENTS

There has also been substantial research examining sex differences in pain using rodent models. Studies of basal sensitivity to laboratory-induced pain in rats and mice report an overall greater sensitivity in females (for review: Mogil et al., 2000). But as with human studies, many other variables, such as the testing modality, strongly influence reported sex differences in pain threshold and sensitivity. Most studies measuring responses to electrical stimulation report greater female sensitivity (Beatty & Beatty 1970, Beatty & Fessler 1977, Pare 1969), whereas thermal assays, such as the hot-plate and immersion of the tail in heated water, variously find greater sensitivity in females (Grisel & Mogil 2000), males (Bartok & Craft 1997, Craft et al., 1999) or neither (Grisel & Mogil 2000, Kavaliers & Colwell 1991).

1.6.1. Influence of menstrual/estrus cycle on pain sensitivity in rodents

Hormonal changes in free-cycling females have been suggested to induce anatomical and physiological changes that lead to altered behavioral sensitivity. In women, levels of progesterone and estrogen change over a 24-30 day cycle. Both hormones increase during the follicular phase after the hypothalamus signals the pituitary gland to release follicle-stimulating hormone. This culminates in a peak of estrogen and progesterone just prior to ovulation. Progesterone levels peak again during the luteal phase, but estrogen levels decrease. Both hormones drop toward the end of the luteal phase and remain low during menstruation. In rodents a similar cycle occurs over a four-day span. During proestrus, estrogen and progesterone levels are both high and slowly decline during estrus. While estrogen continues to drop, progesterone peaks again during diestrus.

Studies of the impact of estrus on behavioral sensitivity are not conclusive but do point to measurable variability across the cycle. In proestrus when both estrogen and progesterone levels are relatively high, female rats demonstrate more behavioral hypersensitivity relative to males as well as to females at other points in their cycle (Bradshaw et al., 2000). Uterine primary afferents are more sensitive to distension (Everitt & Robbins 1992), while the threshold for responses to colorectal distension is significantly lowered (Ji et al., 2008).

The impact of estrus phase on pain sensitivity may not be universal, however, and may vary with the testing modality and other methodological factors such as strain. Sex differences in the magnitude of pain behaviors in formalin-evoked pain are not estrus-dependent (Vincler et al., 2001), and Mogil et al., (2000) reported that estrus-dependent differences in thermal sensitivity with Swiss Webster mice are not present in the CD-1 strain.

1.6.2. Impact of exogenous ovarian hormone on pain sensitivity in rodents

Gonadectomy (GDX) in rodents, with or without supplemental hormone replacement, has been used to examine the effects of ovarian hormones on nociceptive sensitivity. GDX involves the surgical removal of the testes in males and the ovaries in females. Kaur et al., (2018) report that peripheral serotonin-evoked pain was more pronounced for females in proestrus and estrus compared to males and ovariectomized (OVX) females, suggesting that states of high endogenous estrogen predispose females to some forms of behavioral sensitivity. In many cases, GDX, which eliminates the source of ovarian hormones, diminishes or abolishes behavioral differences in sensitivity between the sexes. In one study, merely blocking the target of ovarian hormones similarly resulted in the elimination of sex differences; after ablation of estrogen receptors α or β, mechanical hypersensitivity was not significantly different between male and female mice (Li et al., 2009).

Treatment with exogenous estrogen alters various forms of hypersensitivity, sometimes reversing the antinociceptive effects of GDX. Supplemental estradiol alters the activity of the hypogastric nerve after cervical distension (Liu et al., 2005), and modulates the visceromotor reflex and responses of spinal dorsal horn neurons to colorectal stimulation in rats. Additionally, OVX females who received exogenous estradiol showed more visceral hypersensitivity than vehicle-treated rats following a forced-swim paradigm (Hubbard et al., 2016). But other studies have pointed to a more complex picture of the effect of estrogen on pain, indicating opposing effects at high and low doses (Craft et al., 2008). Supplemental estradiol after GDX actually reduced nociceptive behaviors in female rats that had been treated with formalin in the hind paw (Mannino et al., 2007). This argues that estrogenic activity protected against, rather than potentiated, increased hypersensitivity. Given the wide distribution of estrogen receptors in the periphery, spinal cord and brain (Papka et al., 2001, Vanderhorst et al., 2002), estrogenic effects in pain processing are likely mediated at many levels, with potentially conflicting effects.

1.7. SEX DIFFERENCES IN NOCICEPTIVE THRESHOLDS IN RODENTS WITH PERSISTENT PAIN

In some cases, sex differences in mechanical hypersensitivity only emerge after the induction of a pain state. Females with basal mechanical sensitivity equivalent to their male counterparts exhibited greater hypersensitivity after the induction of persistent inflammation, such as after the injection of Complete Freund's Adjuvant (CFA) (Bradshaw et al., 2000). Differences in behavioral measures of pain sensitivity in the formalin test (another model of persistent inflammatory pain) are also well documented and fairly consistent (Grisel & Mogil 2000, Nazarian et al., 2014).

Rodent strain nonetheless appears to have a significant impact on whether there are sex differences in the development of persistent pain. After spinal nerve transection,

Sprague-Dawley (SD) females have greater levels of hypersensitivity than SD males, but there were no sex differences in mechanical thresholds with Holtzmann rats (DeLeo & Rutkowski 2000). Similarly, the magnitude of mechanical allodynia (a form of hypersensitivity defined by the experience of "pain due to a stimulus that does not normally evoke pain" (Vervest & Schimmel 1988)) in lumbar radiculopathy is sex and strain-dependent (LaCroix-Fralish et al., 2005).

Regardless of whether there are differences in the magnitude of hypersensitivity, female rodents in models of chronic pain do appear to develop hyperalgesia (a hypersensitivity defined by the experience of "increased pain from a stimulus that normally provokes pain" (Vervest & Schimmel 1988)) and allodynia earlier than males, and often display lower pain thresholds after the emergence of pain (Dominguez et al., 2012, Gregory et al., 2013, Tajerian et al., 2015). Hypersensitivity is also reported to resolve more slowly in females (Nicotra et al., 2014, Vacca et al., 2014). Thus, while there is room for debate as to whether healthy females are truly more sensitive to laboratory-induced pain, like their human counterparts, female rodents with persistent pain appear to experience greater nociceptive sensitivity, at an earlier stage, and often for much longer.

1.8. ENDOGENOUS PAIN MODULATION

Nociceptive information from the periphery is modulated continuously at the level of the spinal cord in response to information about behavioral needs, pathological states, and signals from the external environment. These messages, mediated by supraspinal regions via an output from the brainstem, can inhibit or facilitate pain. Clinical and laboratory tests of endogenous pain modulation reveal that many pain patients exhibit either reduced pain inhibition (King et al., 2009) or enhanced pain facilitation (Edwards et al., 2003, Fillingim et al., 1998, Maixner et al., 1998) relative to pain-free controls. As

outlined below, defects in endogenous pain modulation are thought to underlie the pathophysiology of many chronic pain states (Aderjan et al., 2010, Basbaum & Fields 1978, Jensen et al., 2009, Johannesson et al., 2007, Julien et al., 2005, Kosek & Ordeberg 2000, Lannersten & Kosek 2010, Lautenbacher & Rollman 1997, Staud 2009, van Wijk & Veldhuijzen 2010, Wilder-Smith et al., 2004, Witting et al., 2003).

1.9. SEX DIFFERENCES IN ENDOGENOUS PAIN MODULATION IN HUMAN SUBJECTS

Conditioned-pain modulation (CPM) refers to laboratory-induced pain assessed by a concurrently presented second noxious stimulus. Defects of "pain-inhibited pain" have been associated with many chronic pain conditions including migraine, IBS (Brinkert et al., 2007, Piché et al., 2011) fibromyalgia (Lautenbacher & Rollman 1997), and complex regional pain syndrome (Seifert et al., 2009). Notably, although deficiencies in CPM are common in pain syndromes with a female patient majority, many evaluations of CPM in the laboratory setting reveal no basal sex differences (Baad-Hansen et al., 2005, Lautenbacher et al., 2008). Nonetheless, in a systematic review of sex differences in CPM, Popescu et al., (2010) found that a majority of studies using pain report as the measure found more efficient CPM in males than in females, though the relative magnitude of the effect varied greatly depending upon the experimental methodology.

Temporal summation is a psychophysical assessment used as a measure of pain facilitation. It was initially observed in neuropathic pain patients that repeated innocuous stimuli could become painful, and that previously noxious stimuli could become more painful still. This led to the development of tests of temporal summation in which a single stimulus, for example an innocuous poke, is applied to the same part of the subject's body repeatedly. If the rate of application is slow enough, the subject will typically report a consistent sensation intensity from poke to poke. However, if the rate of application is increased, the reported intensity of the sensation will increase from one stimulation to

the next, and can become painful. Temporal summation using a noxious stimulus was also found to occur in pain-free subjects, and can be evoked by repeated stimulation with a noxious heat at a rate of at least 0.33 Hz (Vierck et al., 1997). Enhancements of temporal summation have been reported in a variety of pain conditions, including those that are more prevalent among women such as IBS (Berman et al., 2000) and fibromyalgia (Staud et al., 2001). Fibromyalgia patients additionally show less pain inhibition (by conditioned pain modulation) of temporal summation than healthy controls (Staud et al., 2003). Enhancements in temporal summation have also been seen in pain-free women, relative to their male counterparts (Fillingim et al., 1998, Sarlani et al., 2004, Sarlani & Greenspan 2002) and match those seen in women with chronic pain (Staud et al., 2003). This latter finding suggests that some of the difference in temporal summation between pain patients and healthy controls for disorders more prevalent in women may in fact be due to basal differences in pain modulation between the men and women.

1.10. SEX DIFFERENCES IN ENDOGENOUS PAIN MODULATION IN RODENTS

Rodent studies of CPM (termed Diffuse-noxious inhibitory control (DNIC) with non-human subjects) and temporal summation have permitted insight into the mechanisms of these phenomena. But there are comparatively few inquiries into whether there are sex differences in DNIC or temporal summation. Da Silva et al., (2018) reported that DNIC was greater in intact males than in either females or GDX males, suggesting that testosterone may a role in this form of endogenous pain modulation. Lomas & Picker (2005) reported that males exhibited slightly greater temporal summation than females, in contrast to research with human studies. However, the use of qualitative pain measures (reported pain intensity) in human studies versus quantitative measures in rodent studies (changes in latencies to withdrawal) may be a critical factor in explaining this difference. This also suggests top-down influences such

as levels of anxiety may interact with the mechanisms of temporal summation in a sex-specific or species-specific manner. A thorough investigation of sex differences in the circuits of pain processing and modulation has yet to be completed.

1.11. Mechanisms of endogenous pain modulation and central sensitization

As noted above, the perception of pain is continuously modified in response to behavioral needs, pathological states and environmental pressures. This interplay of supraspinal top-down inputs that relay information about exteroceptive (relating to sensations that arise in response to stimuli external to the body such as sight and smell) and interoceptive states (relating to sensations that arise from internal receptors such as those that detect core body temperature), and bottom-up nociceptive information from the periphery, alters pain perception via mechanisms at the level of the spinal cord. These include changes in synaptic strength (Ji et al., 2003a), local interneuron effects

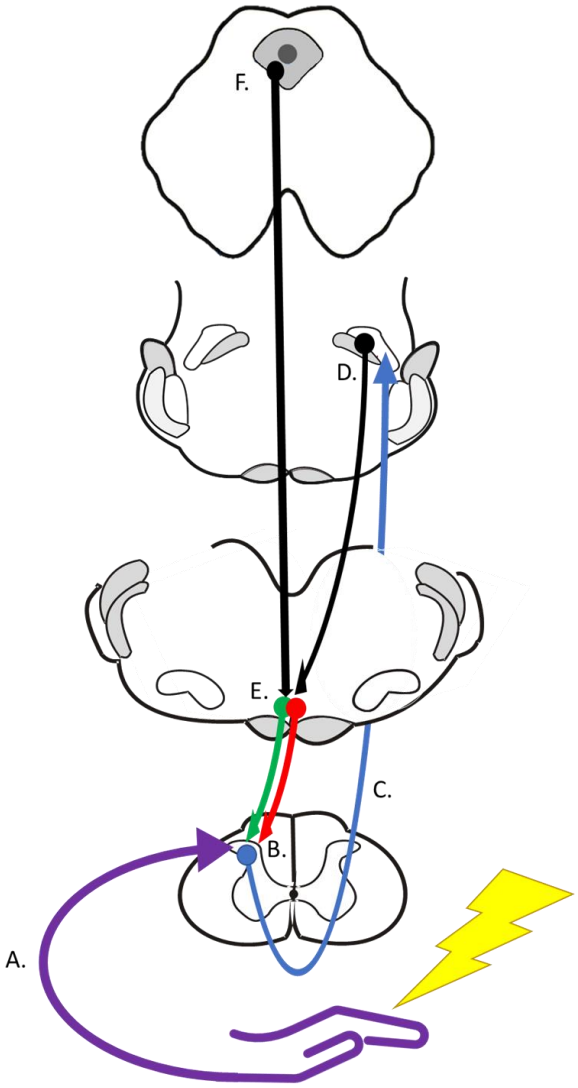

Figure 1: Organization of the descending pain modulating circuitry

A. Primary afferent
B. Dorsal horn neuron
C. Ascending afferent
D. Parabrachial nucleus
E. Rostral ventromedial medulla
F. Periaqueductal gray

(Kim et al., 2012), and input from the RVM which acts as the output node of the descending pain-modulating circuitry (Gilbert & Franklin 2001, Tillu et al., 2008).

1.11.1. Organization of the descending pain modulating circuitry

Acute nociceptive pain is initiated in the periphery with activation of C- and Aδ-fibers (fig.1A). These fibers synapse onto secondary neurons in the spinal dorsal horn (fig.1B), the majority of which have axons that cross the midline before ascending to supraspinal recipients of nociceptive input (fig.1C), like parabrachial complex (fig.1D), the RVM (fig.1E), and periaqueductal gray (PAG) (fig.1F). In turn, many of these supraspinal targets of ascending sensory input project back to the dorsal horn where they can modulate the activity of nociceptive neurons and alter the transmission of nociceptive information.

One important component of this network, the PAG-RVM circuit, was recognized in part from electrical stimulation studies that demonstrated pronounced antinociception after focal stimulation of the PAG (Aimone et al., 1987, Barbaro 1988, Nichols & Thorn 1990) or RVM (Zhuo & Gebhart 1997). Later work connected the PAG, and specifically the ventrolateral sub-region of the PAG (vlPAG), with analgesia induced by acute stress (Rosen et al., 1992, Siegfried & de Souza 1989) and the action of systemic opioids (Bodnar 2000, Manning & Franklin 1998, Ossipov et al., 1995). Neurons within the vlPAG synapse directly onto pain modulating neurons within the RVM. Pharmacological or optogenetic manipulations of these neurons alters the firing pattern of RVM neurons and can alter behavioral sensitivity (Morgan et al., 2008). Although the PAG has a small, direct projection to the dorsal horn, most of its effect on nociception is mediated through the RVM.

Pharmacological and stimulation studies established the RVM as the output node of this descending pain-modulating circuitry. As a small brainstem region that includes the raphe magnus and bordering reticular region, the RVM receives multiple bottom-up

and top-down inputs. Ascending nociceptive information is transmitted from the dorsal horn to the lateral parabrachial nucleus (lPB), where Calcitonin gene-related peptide negative (CGRP(-)) neurons send axons that synapse with pain-modulating neurons in the RVM while information about the external environment or internal affective states arrives from higher centers such as the hypothalamus, amygdala and PAG. Although the early stimulation studies pointed to a pain-inhibiting role for the RVM, later work has shown that the RVM exerts a bidirectional effect on pain (Fields & Heinricher 1985). This effect includes the modulation of nociceptive tone continuously based upon behavioral needs, emotional and pathological states, and environmental cues, thus maintaining a balance between pain-inhibition and -facilitation. This parallel facilitation and inhibition of pain is accomplished via diffuse projections from the RVM to layers I, II and V of the spinal dorsal horn (Fields et al., 1995), where close functional relationships between the output of RVM neurons

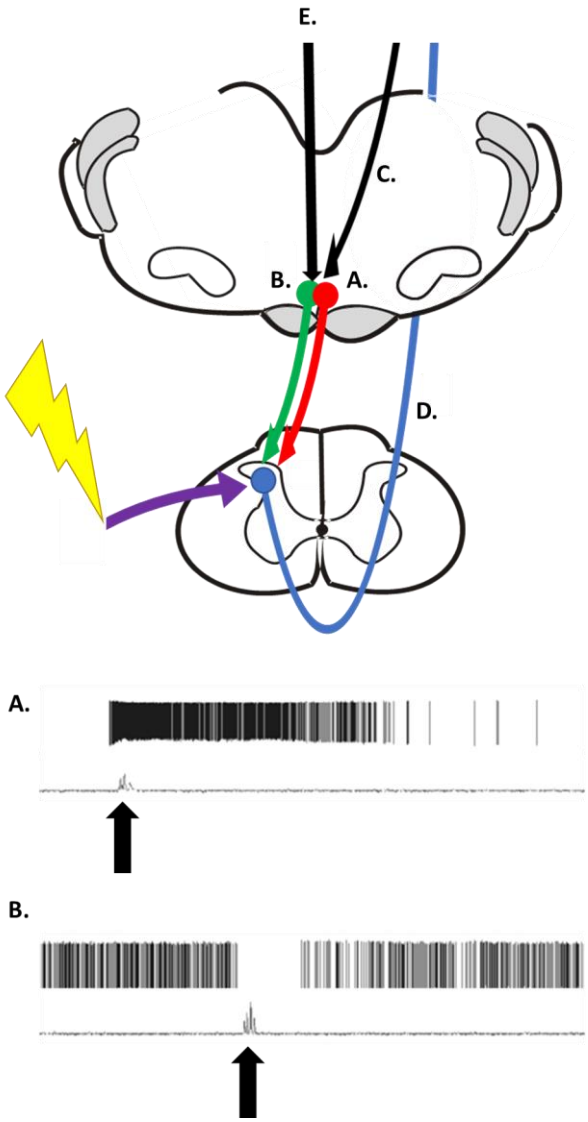

Figure 2: Physiology of the RVM
ON-cells (A) fire a burst of action potentials at the moment of withdrawal (marked with a black arrow), while OFF-cells (B) pause their firing. Neurons in the lPB (C) receive ascending input (D) and directly synapse onto both ON- and OFF-cells, as do neurons in the PAG (E).

and nociceptive neurons in the spinal cord mediate behavioral alterations in sensitivity (Salas et al., 2018, Salas et al., 2016).

1.11.2. Physiology of the RVM

The RVM facilitates and inhibits nociceptive transmission by the action of two neuron classes: ON cells, which exhibit a burst of action potentials in response to a noxious stimulus (fig.2A), and OFF-cells, which respond with a pause in their ongoing firing (fig.2B). A third class of neurons in the RVM, termed NEUTRAL cells, is defined by the absence of any change in firing in response to noxious stimuli. It is unknown if the latter have a role in pain modulation (Fields & Heinricher 1985). Potentially tissue-damaging sensory information reaches these pain modulating neurons in the RVM from neurons in the lPB (fig.2C) that receive ascending nociceptive information from the spinal cord (fig.2D), as well as other interoceptive and exteroceptive information from other spinal and supraspinal sources. Direct projections from the vlPAG to RVM neurons (fig.2E), although unlikely to be a source of noxious input to the RVM can influence the output of the RVM through modulation of both the ongoing and evoked activity of ON- and OFF-cells. RVM neurons project to the superficial dorsal horn, where they alter nociceptive transmission.

The activation of ON-cells facilitates nociceptive transmission in the dorsal horn. In response to noxious input, ON-cells, if previously silent, will emit a burst of action potentials just prior to a nocifensive withdrawal (fig.2A). ON-cells fire together at the population level, and this activity alters the sensitivity of the system to the next input. The threshold required to elicit a paw withdrawal for a noxious thermal stimulus that occurs during the ON-cell burst is slightly lower than when the same stimulus occurs while ON-cells are quiet (Heinricher et al., 1989). Unsurprisingly, changes in the activity of ON-cells are associated with some forms of hyperalgesia (Heinricher et al., 2009, Neubert et

al., 2004) and pharmacological manipulations that selectively target ON-cells are sufficient to elicit behavioral sensitivity.

In contrast, OFF-cells have a net anti-nociceptive effect and pause their ongoing activity in response to a noxious stimulus (fig.2B). As OFF- and ON-cells fire out of phase, it was assumed that ON-cells may behave as local inter-neurons whose burst of activity permits the OFF-cell pause to occur. Cleary et al.,(2008) showed, however, that the OFF-cell pause always precedes the ON-cell burst. Furthermore, while the OFF-cell pause is required for withdrawal, elimination of the ON-cell burst is not sufficient. Elimination of the OFF-cell pause with systemic or local administration of mu-opioid agonists, for example, produces antinociception (Heinricher & Ingram 2008, Heinricher et al., 2010b, Heinricher et al., 1994).

1.11.3 Plasticity in the RVM following persistent inflammation

Strong evidence suggests that behavioral hypersensitivity in persistent inflammation or following nerve injury is due, in part, to an increase in ON-cell output and/or decrease in OFF-cell output (Cleary & Heinricher 2013a, Heinricher & Fields 2013, Heinricher et al., 2010a, Heinricher et al., 2009, Porreca et al., 2002). Behavioral hyperalgesia in the first hour following a localized inflammatory stimulus, such as an injection of CFA in the plantar surface of one hind paw, is accompanied by a dramatic shift in RVM activity, with ON-cells exhibiting an increase in both ongoing and evoked activity while OFF-cell firing is depressed. Under these conditions of ON-cell hyperactivity, inactivation of RVM interferes with behavioral hyperalgesia. Throughout this period the firing of the NEUTRAL-cells does not change (Chen & Heinricher 2019, Cleary & Heinricher 2013a).

The behavioral hypersensitivity and alterations in ON- and OFF-cell activity in persistent pain states are accompanied by molecular, cellular, and pharmacological changes in the RVM circuit. Local inflammation of the hind-paw leads to changes in

neurotransmitter release and neurotransmitter receptor expression and function, including opioid receptors (Guan et al., 2004, Guan et al., 2003a, Guan et al., 2002, Hurley & Hammond 2000, LaGraize et al., 2010, Schepers et al., 2008, Zhang et al., 2011). Concomitantly, the efficacy of opioid agonists microinjected directly into the RVM increases (Hurley & Hammond 2000, Hurley & Hammond 2001). The analgesia induced by electrical stimulation has also been observed to be initially attenuated, but then increase steadily over the first day in response to induction of peripheral inflammation (Guan et al., 2003a, Guan et al., 2002, Terayama et al., 2000).

1.12. SEX DIFFERENCES IN THE NEURAL SUBSTRATES OF PAIN TRANSMISSION AND MODULATION

Interactions between sex and the circuits involved in pain transmission and modulation may contribute to sex differences in pain sensitivity in healthy individuals, as well as in those experiencing chronic pain. Activational and pharmacological differences between males and females have been identified in both ascending and descending pain pathways.

1.12.1. Sex differences in ascending nociceptive processing

Some differences in pain threshold and tolerance may be due to sexual dimorphism in peripheral nociceptive processing. The release of substance P (a pronociceptive polypeptide) in the spinal cord from primary sensory afferents is greater in intact females than males, and is dependent upon gonadal status (Herrero et al., 2000). The release of substance P is thought to play a critical role in wind-up, an amplification of sensory transmission that involves sensitization of secondary neurons of the dorsal horn (Herrero et al., 2000). Female sex appears to result in other mechanisms of amplification at the spinal level. N-methyl-D-aspartate (NMDA)-induced excitatory currents in the dorsal root ganglia (DRG) were greater in female rats than in males.

Neurons in the spinoparabrachial pathway, which is an important source of ascending nociceptive information not only to the RVM, but also to other supraspinal areas potentially mediating sex differences in pain, were more activated in males by a painful visceral stimulus than in females (Murphy et al., 2009), and had activity that was suppressed by morphine to a much greater extent. Counterintuitively, DRGs innervating the gastrocnemius muscle from female rats have a more hyperpolarized resting membrane potential, and C-fibers innervating the muscle have a higher mechanical threshold (Hendrich et al., 2012) even though females tend to withdraw more quickly, or at a lower force, in response to stimuli that activate C-fibers (Grisel & Mogil 2000) Additionally, activity-dependent slowing (ADS) in these fibers, defined as a change in the conduction velocity of action potentials in response to repetitive stimuli, is greater in females (Dickie et al., 2017). Because reductions in ADS facilitate spinal summation (ADS can be thought of as a brake, adding distance between successive action potentials so they are less likely to arrive at the spinal synapse at the same time) it could be reasonably assumed that the greater ADS recorded in female neurons would make them less susceptible to this form of pain facilitation. However, this is not the case: females are generally more behaviorally sensitive than males. It is likely that amplification of pain in the periphery is not sufficient to explain sex differences involving spinal and supraspinal mechanisms.

1.12.2. Sex differences in descending nociceptive processing

The PAG, a midbrain structure with topographically distinct sub-regions receives dense afferent projections from spinal areas relaying nociceptive transmissions and higher centers such as the anterior cingulate cortex (ACC) and amygdala (Bandler et al., 1991, Bandler & Keay 1996, Odeh & Antal 2001, Vanderhorst et al., 1996). The PAG plays a role in multiple behavioral responses, including stress-induced analgesia and escape behaviors (Bandler & DePaulis 1991, Behbehani 1995, Bodnar et al., 1980). One

sub-region, the vlPAG, also significantly contributes to the antinociceptive effect of opioid drugs (Bodnar 2000, Jacquet & Lajtha 1976, Jensen & Yaksh 1989, Tershner et al., 2000), as well as the change in their efficacy after the induction of persistent pain (Wang et al., 2006). Additionally, neurons in the PAG that project to the RVM also display prominent sexual dimorphism. vlPAG-RVM projecting neurons in males are more activated by inflammation (Loyd et al., 2007) and GABAergic signaling in these neurons is altered by inflammatory pain in a sex-dependent manner (Tonsfeldt et al., 2016). Direct modulation of GABA signaling in either the vlPAG or RVM can alter nocifensive responses (Heinricher et al., 1991, Moreau & Fields 1986, Peng et al., 1996, Sandkühler et al., 1991) and these changes in GABA signaling, which modulate the tonic inhibition of the descending pain modulating circuitry, may underlie the development of behavioral sensitivity during the transition to chronic pain in the absence of ongoing tissue injury.

1.13. SEXUAL DIMORPHISM IN THE ROSTRAL VENTROMEDIAL MEDULLA

Interest in understanding the neural substrates of observed sex-differences in pain sensitivity has led to the identification of sexual dimorphism in relays that transmit nociceptive information to the RVM. Ascending nociceptive information is relayed to the RVM via the parabrachial complex, another brainstem region which receives extensive information about interoceptive states including hunger, salt intake, and hypoxia (Carter et al., 2015, Davern 2014, Sammons et al., 2016, Yokota et al., 2015). Meanwhile, descending inputs from the PAG, ACC, and central nucleus of the amygdala (CeA) provide potential circuits for information about environmental threats and affective states to be integrated with nociception. As a result, an individual's behavioral output in response to noxious stimulation is intimately tied to changes in the physiological responses of pain-modulating neurons of the RVM.

Still, despite the important role of changes within the RVM in the transition to persistent pain, whether there is sexual dimorphism of the RVM pain-modulating neurons is unknown. Nonetheless, several lines of evidence suggest that the RVM, like the PAG, may also differ between men and women. For example, opioid efficacy in the RVM tends to be greater in males, with microinjections of morphine eliciting more powerful antinociception in males than in females (Boyer et al., 1998), while microinjections of the κ-opioid receptor (KOR) agonist U69593 into the RVM produce mild antinociception in females without altering male tail-flick latencies at all (Tershner et al., 2000). The distribution of KORs on RVM neurons also varies across the estrus cycle (Drake et al., 2007), and because there is significant aromatase expression within the RVM (Gao et al., 2017) that may contribute to estradiol-induced μ-opioid receptor (MOR) internalization, these behavioral and pharmacological differences may result from significant interaction between opioid and sex-hormone signaling.

To date, only two studies have recorded from RVM pain-modulating neurons in females (Craft et al., 2004, Rojas-Piloni et al., 1998). After twenty years of research into the role of the RVM in pain modulation and analgesia, and almost ten years after the identification of the two classes of pain-modulating neurons, the first published work of RVM neurons from female subjects, Rojas-Piloni et al., (1998), found that mechanical stimulation of the uterine cervix increased the firing rate of OFF-cells and decreased the ongoing firing rate of ON-cells, suggesting that such stimulation induces an anti-nociceptive state in the RVM. It was another six years before any researcher addressed stimulus-evoked neuronal responses in neurons recorded from female subjects: Craft et al., (2004) reported that in OVX females given supplemental estradiol, the magnitude of the change in ON- and OFF-cell activity was blunted (i.e., ON-cells still elicited a burst response, but the burst was smaller) compared with that seen in OVX females given placebo. Both studies indicate that the RVM, a critical site of pain modulation within the

descending pain-modulating circuitry, is altered by female sex, but many questions remain. Is the effect of estradiol (supplemental or endogenous) on the evoked firing of RVM neurons large enough to result in a sex difference in behavior? Would this sex difference remain after OVX? Moreover, as neither study assessed RVM cell activity within a pain state, it is unknown whether persistent inflammation produces the same changes in the activity of ON- and OFF-cells in females that are seen in males. Although significant inferences about the function and physiology of RVM neurons in females can be deduced from research done with males, resolving the reasons for the greater burden of chronic pain in women is limited without their inclusion as subjects.

1.14. SUMMARY

There is an acknowledged gender gap in clinical pain. Significantly more women seek and receive treatment for chronic pain conditions, and are more likely to develop chronic pain and experience greater pain spread. In light of this, there is substantial interest in determining what the neurobiological substrates of sex differences in pain are. One approach has been to evaluate the possibility of differences in basal sensitivity. However, the results have been inconclusive. Some studies in humans of laboratory-induced pain report greater sensitivity in women to specific types of painful stimuli, but it remains unclear to what degree these differences are mediated by biological substrates, psychosocial factors, or both. Although studies with rodents are similarly affected by methodological factors such as strain and the choice of noxious stimulus, they have provided insights into potential biological mechanisms of sex differences in pain. The wide distribution of estrogen receptors in the periphery, spinal cord and brain (Papka et al., 2001, Vanderhorst et al., 2002), suggests that estrogenic effects in pain processing could be mediated at many points during pain transmission.

An alternative hypothesis for sex-differences in clinical pain is that differences arise due to sexual dimorphism in the mechanisms of pain modulation or due to differential defects in pain modulation. Some studies with rodents report differences in pain sensitivity that only emerge after the induction of a pain state. Additionally, female rodents with persistent pain, like their human counterparts, appear to experience greater nociceptive sensitivity, at an earlier stage, and often for a longer duration, further implicating the mechanisms of endogenous pain modulation in sex differences in chronic pain.

Pain is continuously modified in response to behavioral needs, pathological states and environmental pressures. A mix of top-down and bottom-up nociceptive inputs alter pain perception via mechanisms at the level of the spinal cord. The output node of the descending pain-modulating circuitry (Gilbert & Franklin 2001, Tillu et al., 2008) is the RVM, via two neuron classes. However, despite the important role of RVM neurons in setting behavioral sensitivity during persistent inflammatory pain, whether there is sexual dimorphism in RVM neuronal responses is unknown.

In this dissertation, I used single-neuronal recording in lightly anesthetized male and female rats to test the hypothesis that there are basal differences in the physiology of pain-modulating neurons in the RVM that contribute to sex differences in pain. Using naive rats of both sexes, I characterize the responses of ON- and OFF-cells in the RVM of males and females in response to noxious thermal and mechanical stimuli. To evaluate whether changes in these pain-modulating neurons occurs with persistent inflammation, I also tested the nociceptive sensitivities and associated neuron activity in males and females after unilateral injections of a pro-inflammatory agent in the hind-paw.

CHAPTER 2

Functional characteristics of RVM pain-modulating neurons in male and female rats with persistent inflammatory pain

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2.1. ABSTRACT

The development of chronic pain is often related to defects in endogenous pain modulating systems, with many changes occurring in the rostral ventromedial medulla (RVM), the output node of the descending pain-modulating circuit. The RVM can facilitate or suppress nociception through the activity of two neuron classes: pro-nociceptive ON-cells and anti-nociceptive OFF-cells. In male rats, the firing characteristics of these neuron classes are altered after the induction of persistent hind paw inflammation. The goal of the present study was to characterize ON- and OFF-cell responses in females, and to assess whether they differ from males.

To determine whether there are differences in RVM neuronal activity between male and female rats, nociceptive thresholds and associated ON- and OFF-cell activity were recorded in lightly anesthetized rats. Thermal latencies and mechanical nociceptive thresholds and associated ON- and OFF-cell firing characteristics were similar between the sexes. Naïve males had shorter withdrawal latencies to a noxious for a mechanical stimulus. Because the firing properties of ON- and OFF-cells in females had not been previously characterized in a pain state, behavioral testing and neuronal recordings were performed in female and male rats 3-6 d after hind paw injections of complete Freund's adjuvant (CFA). Female rats developed robust mechanical (but not thermal) hyperalgesia in the CFA-treated paw, and threshold for ON- and OFF-cells responses was in the innocuous range. However, there were no detectable sex differences in behavioral thresholds or firing characteristics.

These findings suggest that despite sex differences in other regions of the descending pain modulating circuitry, the firing properties and responsiveness of identified pain-modulating neurons in the RVM do not differ between the sexes.

2.2. INTRODUCTION

Chronic pain is a significant cause of suffering and disability (Andrews et al., 2018, Blyth et al., 2019) with differing impacts on men and women. This sex difference has led to increased interest in understanding the interactions of sex and pain. Whether basal pain thresholds or perception differ between males and females remains contested (Racine et al., 2012), and variables in experimental design (such as the pain assay) may strongly influence the reporting of sex differences. However, several studies in rodents showed that differences in sensitivity between males and females emerge only after the induction of a pain state, which would be in line with the greater prevalence of chronic pain in women. For example, female rodents in some models of chronic pain appear to develop hyperalgesia and allodynia earlier, and display lower nociceptive thresholds after the emergence of hypersensitivity (Bradshaw et al., 2000). Hyperalgesia and allodynia also resolve more slowly in females than males (Nicotra et al., 2014, Vacca et al., 2014). Such contrasts in the development and magnitude of hypersensitivity raise the possibility that there are differences in the mechanisms of endogenous pain modulation in males and females.

Nociceptive information from the periphery is modulated continuously in the spinal cord as a function of behavioral needs, pathological states, and signals from the external environment. These influences are mediated by recruitment of the descending pain-modulating circuitry, which can both inhibit or facilitate pain. The development of chronic pain is increasingly recognized as a shift to a pathological state of endogenous pain modulation (Aderjan et al., 2010, Basbaum & Fields 1978, Jensen et al., 2009, Johannesson et al., 2007, Julien et al., 2005, Kosek & Ordeberg 2000, Lannersten & Kosek 2010, Lautenbacher & Rollman 1997, Staud 2009, van Wijk & Veldhuijzen 2010, Wilder-Smith et al., 2004, Witting et al., 2003) with increasing nociceptive tone (Sevcik et

al., 2004) that outlasts the initial injury, or occurs in the absence of obvious tissue damage.

Brain circuits relevant to endogenous pain modulation have been identified. The RVM is the output node of this descending pain-modulating circuit (fig.1) (Gilbert & Franklin 2001, Tillu et al., 2008), and alters nociceptive transmission by the action of two neuron classes (fig. 2A,B): ON-cells, which elicit a burst of action potentials associated with behavioral responses to a noxious stimulus, and OFF-cells, which respond with a pause in their ongoing firing (Heinricher et al., 1989), Strong evidence suggests that behavioral hypersensitivity in persistent inflammation or following nerve injury results, in part, from an increase in ON-cell output and/or decrease in OFF-cell output (Cleary & Heinricher 2013a, Cleary et al., 2008). The behavioral hypersensitivity and alterations in ON- and OFF-cell activity in persistent pain states are accompanied by molecular, cellular, and pharmacological changes in the descending circuit (Guan et al., 2004, Guan et al., 2003a, Guan et al., 2002, Hurley & Hammond 2000, LaGraize et al., 2010, Schepers et al., 2008, Zhang et al., 2011).

Whether there is sexual dimorphism in the physiology of pain-modulating neurons remains understudied, but several lines of evidence suggest that neuronal responses in the RVM may differ between males and females. Neurons in the spinoparabrachial pathway, an important source of ascending nociceptive information to the RVM, are more activated by a noxious stimulus in males than in females (Murphy et al., 2009), as are neurons in the PAG (Loyd & Murphy 2006). Additionally, inflammation activates vlPAG-RVM projecting neurons more in males (Loyd et al., 2007), and alters GABAergic transmission in a sex-dependent manner (Tonsfeldt et al., 2016). There, changes in GABA function, which can modulate ON- and OFF-cell responses, may underlie the development of behavioral sensitivity. Yet, no studies have compared the physiology of RVM ON- and OFF-cells between sexes. In fact, only two studies have

recorded RVM pain-modulating neurons in females (Craft et al., 2004, Rojas-Piloni et al., 1998). However, neither study compared activity in females with that of males, nor assessed RVM cell activity in females with persistent pain.

The purpose of these experiments was to systematically test whether there are differences in pain behaviors and associated cell activity in the RVM between males and females, before and after the induction of persistent inflammation. I found that naive males and females had similar thermal withdrawal thresholds, but mechanical withdrawal thresholds were significantly longer in females. RVM neuronal activity was not significantly different between the sexes under basal conditions. Additionally, females displayed mechanical hypersensitivity that was not significantly different to males after the induction of persistent inflammation, and there were no differences in the responses of RVM neurons.

2.3. Materials and Methods

2.3.1. Subjects

A total of 52 male (250-400g) and 54 female (175-250g) Sprague-Dawley rats (Charles River labs) were used. Rats were housed in groups of 2-3 with a 12-h light: dark cycle (lights on at 6:00 am) with ad libitum access to food and water. Rats were singly-housed after induction of inflammation in the hind-paw. All procedures followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Charlton 1995) and were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University

2.3.2. Inflammation

Rats were anesthetized with isoflurane (4% for 4-5 mins). Complete Freund's adjuvant (CFA, heat-killed Mycobacterium tuberculosis in mineral oil, 0.1ml of 1 mg/1 ml,

Sigma-Aldrich) was injected subcutaneously into the plantar surface of the right hind paw. Animals were returned to their home cages and housed for 3-6 days before the electrophysiological recording session (see fig. 3 for timeline).

2.3.3. Electrophysiological recording

For electrophysiological recording, rats were anesthetized with isoflurane (4%) and a catheter was inserted into the external jugular vein. After transfer to a stereotaxic frame, animals were maintained in a deeply anesthetized state with infusion of the short-acting barbiturate methohexital to allow for drilling of a small craniotomy, and removal of the dura and pia mater. Body temperature was monitored with a rectal probe, and maintained at 36-37.5 °C with a heating pad, while heart rate was measured using electrocardiograph (EKG). Once preparatory surgery was complete, animals were maintained in a lightly anesthetized state by adjusting the methohexital infusion rate (50-70 mg/kg/h) such that there were no spontaneous movements or vocalizations, but hind paw withdrawals could be elicited by a noxious heat or mechanical stimulus. Animals were stabilized at an anesthetic flow rate for at least 30 minutes prior to any data collection.

For extracellular recording, a gold- and platinum-plated stainless steel electrode (Microprobes, Gaithersburg, MD) was used, with signals amplified (10k) and bandpass filtered (400 to 15K Hz) then digitized (32K samples/s). The electrode was placed on the brain surface using visual landmarks, and lowered into the RVM. Neurons were isolated and classified as ON- or OFF-cells by the response associated with nocifensive behaviors (see fig. 7). ON-cells that were previous quiet exhibited a burst of action potentials beginning just prior to the nocifensive withdrawal, or if active, continued to fire. OFF-cells displayed an inverse pattern, with active cells halting firing just prior to paw

withdrawal or if inactive, remaining silent. A third class of neuron in the RVM (NEUTRAL cells) was characterized by the absence of a response to noxious stimulation. Whether NEUTRAL cells play a role in endogenous pain modulation is unclear (Heinricher & Neubert 2004, Kincaid et al., 2006), Cleary et al., (2013) demonstrated that NEUTRAL cell activity remains unchanged in the acute and persistent phases on CFA-induced hind paw inflammation in males. For this reason, NEUTRAL cells were excluded for this study. One to two neurons per rat were studied in each animal.

2.3.4. Nociceptive testing

Once an ON- or OFF-cell was identified, thermal and mechanical thresholds for both the left and right hind-paws were tested. To determine thermal thresholds, the hind paws were placed on a small platform with the plantar surface face-up. A Peltier probe with a holding temperature of 35 °C was gently placed on the plantar surface of the paw. The probe was held in place for 30 s before the temperature ramp was initiated, with a linear increase of 1.2 °C/s. The probe was removed as soon as a response was detected, or at 53 °C in the absence of a withdrawal, to avoid tissue damage. Withdrawals were detected with electromyography (EMG) with electrodes placed approximately 1 cm apart within the gastrocnemius muscle. The EMG signal was rectified and smoothed, and the first positive inflection was defined as the onset of the withdrawal. If a withdrawal was observed visually but was not detected by EMG, then withdrawal data from that trial was excluded. In the absence of visually observed withdrawal that was also not detected by EMG, a cut-off of 10 s was assigned as the response latency. Each paw was tested once, with an interval of 5 min between tests on each side. Mechanical thresholds were determined using von Frey (vF) fibers (4 to 100 g) applied to the webbing between the toes. Each fiber was applied three times to each paw, in ascending order, for 8 s. Three interdigital testing sites were alternated, with a

minimum interval of 30 s between each trial. As with thermal stimulus trials, if a withdrawal was observed visually but not detected by EMG, data from that trial was excluded. If data was missing for two or more trials out of three, data for that fiber and paw combination were excluded. Mechanical thresholds for each paw were assigned according to the minimum force at which a withdrawal was detected by EMG in at least two trials. In the absence of a visually confirmed withdrawal, a latency of 8 s (the duration of the stimulation) was assigned to the trial. Due to the variable spontaneous activity of ON- and OFF-cells, longer inter-trial intervals (up to 5 min) were sometimes necessary to catch ON-cells in an inactive state and OFF-cells in an active state.

2.3.5. Histology

Recording sites in RVM were marked with an electrolytic lesion produced by passing a 7 mA current for 8 s through the electrode. Animals were then euthanized with an overdose of methohexital, intracardially perfused with 0.9% saline and 4% formaldehyde, and the brains were collected and stored in 4% formaldehyde. The RVM was defined as the nucleus raphe magnus and adjacent ventromedial reticular formation medial to the lateral edge of the pyramids at the level of the facial nucleus. Hand drawn sections of the RVM were used to the location (fig. 5) and distribution (fig. 6) of recording sites using landmarks defined by Paxinos & Watson (2009).

2.3.6. Data analysis

The extracellular recording signal, EMG, and EKG were digitized and collected using Spike 2 software (Cambridge Electronics Design Ltd, Cambridge, UK). Each waveform was sorted using Spike2 template matching and cluster analysis and verified offline on an individual spike basis.

Several cell parameters were used to characterize changes in RVM cell activity. Ongoing cell activity was determined from a 30-s interval near the beginning of each

recording, prior to the first heat-stimulus trial. Cell activity associated with the paw withdrawal was assessed by quantifying the peak firing rate in the ON-cell burst as well as the total number of spikes associated with the paw withdrawal. In cases where behavioral withdrawal was noted visually but was not detectable on EMG, no changes were made to the peak firing rate data, but the total number of spikes in the ON-cell burst was not used from that trial and was replaced by the total number of spikes that occurred in during the 8 s window of the trial minus the number of spikes that occurred during the 8s window just prior to the beginning of the trial. For OFF-cells, the duration of the pause in OFF-cell firing was analyzed. For OFF-cells, data from trials in which a withdrawal was confirmed visually but not detectable on EMG were excluded with no replacement.

Statistical analyses were performed in Prism 8 (GraphPad). Unless otherwise noted, data are presented as geometric mean ± SEM. Behavioral data comprised of continuous data from two independent groups was analyzed using a Mann Whitney U test to account for non-normal distributions. Continuous behavioral data with ≥3 groups were log-transformed to permit analysis with parametric tests: data sets with no missing values were analyzed using ANOVAs with *post hoc* Tukey's test; in the case of missing data, a Mixed-effects model (REML) with either a *post hoc* Sidak's multiple comparisons test or Tukey's test was chosen.

Because cell parameters for RVM neurons are typically skewed (neither the number of counted spikes nor the duration of the pause can have negative values), cell parameter data were log-transformed before analysis to permit the use of parametric tests. Continuous cell data (all comparisons had ≥4 groups) were analyzed with ANOVAs in the absence of unequal groups and/or missing values, or a Mixed-effects model (REML). These were followed by *post hoc* Sidak's multiple comparisons test or Tukey's test as noted. For all tests, $p < 0.05$ was considered significant.

2.4. RESULTS

2.4.1. Ongoing activity of RVM ON- and OFF-cells in male and female rats

Neurons in the RVM display frequent and patterned spontaneous activity, which can change in response to acute stimuli, or in early pain states (Cleary & Heinricher 2013a). The spontaneous activity of these cells was measured in males and females by analyzing cell activity prior to behavioral testing. Ongoing firing rates (fig. 8) of ON-cells were not significantly different between males (naïve: 1.40 Hz [95% CI: 0.00, 2.92]; CFA: 3.058 HZ [95% CI: 0.60,5.52]) and females (naïve: 2.16 Hz [95% CI: 0.00,5.66]; CFA: 1.80 Hz [95% CI: 0.21,3.39]). Although ongoing activity of ON-cells increases in the first hour after CFA administration, spontaneous ON-cell activity returns to baseline levels over the course of the first 24 hours (Cleary & Heinricher 2013a). Consistent with this pattern, the ongoing activity of both ON-cells was not significantly altered by treatment with CFA, in either or females. The ongoing firing rates of OFF-cells followed a similar pattern: firing rates between males (naïve: 8.77 Hz [95% CI: 5.31, 12.24]; CFA: 8.70 HZ [95% CI: 2.80, 14.59]) and females (naïve: 9.56 Hz [95% CI: 6.60, 12.52]; CFA: 7.76 HZ [95% CI: 3.60, 11.93]) were not significantly different, and were not altered by treatment with CFA.

2.4.1. Thermal pain thresholds and associated neuron activity in males and females

After comparing spontaneous activity of RVM neurons, we next sought to analyze behavioral thresholds and cell activity associated with nocifensive withdrawals. Thermal nociceptive thresholds were assessed in naive male and freely-cycling female rats using a Peltier probe. After placement on the plantar surface of the paw, the probe was steadily ramped from an innocuous warm temperature to noxious heat, permitting the individual threshold to be determined. Although some reports have indicated sex differences in thermal thresholds with the use of static heat sources, I found that thermal

sensitivities were not significantly different between naïve males and females (males: 50.60 °C [95% CI: 49.90, 51.30]; females: 51.19 °C [95% CI: 50.54, 51.84]). Temperature thresholds were also not significantly altered following CFA treatment (fig. 9A) (males: 51.30 °C [95% CI: 50.70, 51.90]; females: 51.19 °C [95% CI: 50.63, 51.76]). Stimulus-evoked activity, as measured by the firing patterns of RVM neurons to noxious thermal stimuli, was also similar between the sexes. The peak firing rates of ON-cells associated with nocifensive withdrawal (fig. 9B) were not significantly different between males and females (naïve males: 11.25 Hz [95% CI: 8.24, 14.26]; naïve females: 12.80 HZ [95% CI: 7.43, 18.17]), and did not change significantly after treatment with CFA (males: 16.47 Hz [95% CI: 9.99, 22.953]; females: 13.56 HZ [95% CI: 9.61, 17.51]). Likewise, there was no effect of sex or treatment on stimulus-evoked activity of ON-(fig. 9C) (naïve males: 227.083 [95% CI: 0, 529.72] and females: 221.80 [95% CI: 48.932, 394.67] versus CFA males: 338.80 [95% CI: 0, 724.29] and females: 372.50 [95% 27.92, 717.083]), or OFF-cells (fig. 9D) (naïve males: 2.30 s [95% CI: 1.0050, 3.55] and females: 5.56 s [95% CI: 2.12, 9.18] versus CFA males: 5.15 s [95% CI: 2.38, 7.92] and females: 2.16 s [95% 2.16, 16.81]). Altogether, male and female rats had very similar responses to a thermal stimulus, both behaviorally and physiologically.

2.4.2. Mechanical thresholds and associated neuronal activity in naïve males and females

Differences in sensitivity to noxious mechanical stimuli have been reported for healthy males and females (Li et al., 2009), with the magnitude and direction of differences depending upon testing modality. Thus, we were interested in assessing the mechanical sensitivities and associated neuronal responses in females to see if they differed from males. We used vF probes applied to the webbing of the toes to determine the mechanical thresholds. Mechanical nociceptive thresholds were comparable

between the sexes (fig. 10A). However, females had significantly longer latencies to the hind paw withdrawal (fig. 10B) with the 60g fiber (males: 0.48 s [95% CI: 0.38, 0.57]; females: 0.66 s [95% CI: 0.61, 0.72]). To assess whether there were differences in cell responses to mechanical stimulation, we again analyzed the evoked ON- and OFF-cell responses and found that the ON-cell peak firing rate (fig. 11A) and total number of spikes (fig. 12A) in the ON-cell burst increased as the force of the mechanical stimulus increased, but did not differ significantly between males and females. Likewise, the duration of the OFF-cell pause increased with stimulus intensity (fig. 13A) with no difference between male and female rats. The association between evoked RVM neuronal activity and behavioral withdrawal was similar in males and females despite shorter latencies to withdraw in males.

2.4.3. Mechanical thresholds and associated neuronal activity in CFA-treated male and female rats

We next sought to determine whether further differences emerged after induction of a pain state, in this case persistent inflammation. To permit characterization of changes in activity of RVM neurons after the induction of persistent inflammatory pain, CFA was injected into the hind paws of both male and female rats 3-6 days before electrophysiological testing. Consistent with previous reports (Craft et al., 2013) CFA-injections elicit robust mechanical hypersensitivity in both male and female rats. Paw withdrawal thresholds were significantly lower on the injured paw compared to naive control animals (fig. 14A) (naïve males: 58.38 g [95% CI: 55.0040, 61.76] and females: 60 g [95% CI: 60.00,60.00] versus CFA males: 16.61g [95% CI: 13.94, 19.29] and females: 22.033 [95% CI: 15.71, 28.36]). However, there was no significant sex difference between the mechanical thresholds. The changes in withdrawal threshold were accompanied by significantly shorter withdrawal latencies to withdrawal within the

innocuous range (≥ 26 g, fig. 14b) for CFA-treated animals. Although shorter response latencies for withdrawal to vF stimulation have been reported for females (Craft et al., 2013), we saw no effect of sex in CFA-treated rats.

The induction of persistent inflammation significantly altered neuronal responses to both innocuous and noxious mechanical stimuli on the injured side. Treatment with CFA shifted the response curves of RVM neurons to the left into the “innocuous” range, and had a significant effect on the peak firing rate (fig.15A) associated with the nocifensive withdrawal for both males and females. Likewise, the evoked ON-cell activity associated with paw withdrawal on the injured side was significantly greater compared to uninjured rats (fig.16A) or paw withdrawal on the uninjured side (fig.17A) OFF-cell responses associated with responses to stimulation of the CFA-treated paw were also altered. The duration of the pause in the innocuous range was significantly increased for both male and female rats when compared to healthy controls (fig. 18A) and the uninjured paw (fig. 19A). Finally, plotting of latencies to behavioral withdrawal with latencies to burst for ON-cells (fig. 20A), and latencies to pause for OFF-cells (fig. 20B) did not reveal a significant sex effect. Overall, male and female rats had very similar behavioral responses for multiple stimulus modes and had similar associated cell responses both in the naïve state and after the induction of persistent inflammatory pain.

2.5. DISCUSSION

Despite increasing interest in the mechanisms that underlie sex differences in clinical pain, much of our understanding of the neurobiological substrates of pain processing has come from research that exclusively used male subjects. Differences between males and females have been identified in brainstem areas of the descending pain modulating circuitry (Murphy et al., 2009), including areas that relay nociceptive information to the RVM. However, it was unknown whether differences in these inputs

resulted in physiological differences in RVM pain-modulating neurons between males and females. In the present study we characterized RVM neuronal responses of naive female rats as well as females with persistent inflammation to see if responses differed from males. We used a combination of *in vivo* extracellular recording and thermal and mechanical nociceptive behaviors in lightly anesthetized animals. Our results indicate that responses of pain-modulating neurons and nociceptive thresholds in males and females are similar, but that naïve females displayed a significantly longer latency to withdraw to the 60g vF fiber. After the induction of persistent inflammation we did not detect any sex difference in RVM cell activity and mechanical hypersensitivity. Overall this suggests that under certain conditions pain-modulating neurons of the RVM respond similarly to acute pain and in persistent pain between males and females.

When we assessed the spontaneous firing of RVM neurons we found no sex differences in ongoing activity in naïve rats or after CFA-treatment. This is consistent with Craft et al., (2004) who also analyzed activity of RVM neurons in females. They found that supplemental estradiol had no effect on the ongoing firing rates of ON- and OFF-cells sampled from OVX rats when compared with OVX rats given placebo, despite dampening stimulus-evoked activity of the ON-cell burst and OFF-cell pause. This suggests that the influence of estrogenic activity on setting the spontaneous firing of ON- and OFF-cells is minimal, and may not be sufficient to drive a sex difference in firing rate. Additionally, after induction of inflammation we saw no sex difference and no significant effect of CFA-treatment. Cleary et al., (2013) analyzed changes in ON- and OFF-cell activity in the first hour and 3-6 days after CFA-treatment. Their work, and others (Heinricher et al., 1989, Heinricher & Fields 2013, Heinricher et al., 2009), indicates that neurons in the RVM modulate pain sensitivity at a population level: OFF-cells set an antinociceptive tone in the naïve state, while ON-cells set a pronociceptive tone in the first hours after injury (CFA-treatment). In this early pronociceptive state, increases in the

spontaneous activity of ON-cells are responsible for behavioral sensitivity to thermal and mechanical stimuli. Over the next several days, activity in the RVM shifts to a compensatory antinociceptive state in which ongoing activity returns to baseline levels. The results of our study are consistent with this previous data elucidating a relationship between ongoing firing and an antinociceptive state in the RVM, but there remains the possibility that sex differences could emerge during the pronociceptive state just after CFA-treatment. Further studies should explore this.

When we assessed thermal nociceptive responses, we found no sex differences in behavioral and cell responses to thermal stimulation, nor was there a difference after induction of persistent inflammation. Sex differences in thermal sensitivity of naïve rats have been reported by some studies (Bartok & Craft 1997, Craft et al., 2013, Grisel & Mogil 2000, Mogil et al., 2000) but not others (Kavaliers & Colwell 1991, Kavaliers et al., 1998). In rodents, the detection of a sex difference in thermal sensitivity is sensitive to methodological factors such as rodent strain (Mogil et al., 2000) and the type of thermal stimulus used (Hashmi & Davis 2014, Mogil et al., 2000). In one study, Mogil et al., (2000) report a significant sex difference in Sprague Dawley rats (but not in Wistar Kyoto rats), in contrast to our work. However, their choice of thermal stimulus may account for this. Multiple thermal stimuli types are used to determine nociceptive and perceptual thresholds, some of which employ either a "static" heat source (i.e., hot-plate, or a warm water bath) while others use a thermal stimulus that "heats up" through the trial (i.e., Hargreaves or Peltier). Rats in Mogil et al., (2000) were tested using a hot-plate which is warmed to the target temperature, after which rodent subjects are placed on all fours. With a static heat source like this, warming of the skin must occur before heat-sensitive nociceptors are activated, and differences in skin thickness either due to position on the body (i.e., tail versus plantar hind-paw) or between the sexes (Bronaugh et al., 1983) could alter the time-course of that warming (Dirig et al., 1997). This makes

generalizations across methods more difficult and potentially alters the detection of a sex difference. Hashmi & Davis (2014) suggest that sex differences in responses to dynamic rather than static stimuli may help explain some observed perceptual differences in laboratory-induced pain; however, in our study with the Peltier probe we observed no behavioral or cellular differences between the sexes.

The absence of thermal hypersensitivity after treatment with CFA in our study is, however, consistent with previous work in the Heinricher lab (Cleary & Heinricher 2013a) using lightly anesthetized male rodents. This previous work showed transient thermal hypersensitivity in the first hour after CFA-treatment that decreased to baseline levels by the third day after injection (Cleary & Heinricher 2013a). Given the natural variability of behavior, it is possible that a real but small effect of sex on thermal sensitivity could have been missed in our study due to being underpowered to observe such differences. Nonetheless, the behavioral sample sizes we used are larger to accommodate cell data for both neuronal classes, and in the case of the CFA-treated groups they were larger than those in Mogil et al., (2000) who report a significant effect of sex. Where thermal hypersensitivity on the injured side has been reported (Bradshaw et al., 2000), other methodological considerations may account for these conflicts. Bradshaw et al., (Bradshaw et al., 2000) using a radiant heat stimulus observed both thermal sensitivity in CFA-treated rats as well as sex differences in thermal latencies that were significant for females in proestrus, though not overall. It is possible that because our females were intact and free-cycling an estrus-dependent sex-difference was obscured. However, although Craft et al., (2004) did see an effect on the ON-cell burst peak in rats in proestrus versus diestrus 1 or 2, they did not see a concomitant change in behavioral latencies.

In contrast, naïve males and females differed in mechanical sensitivity, with males exhibiting shorter latencies to withdrawal than females. Despite this, there was no

difference in the mechanical thresholds, and RVM neuronal activity was similar between the sexes. The dynamics of the defensive reflex are associated with changes in the physiological responses of pain modulating neurons of the RVM. These cells are defined by characteristic changes in activity: pain-facilitating ON-cells are activated (burst) in response to noxious input while pain-inhibiting OFF-cells correspondingly pause firing just before the nocifensive withdrawal. Although many ON and OFF-cells respond to a variety of noxious and even innocuous stimuli, firing changes are most highly correlated with the presence of behavioral withdrawal, not stimulation (Fields & Heinricher 1985). The OFF-cell pause, which always precedes the ON-cell burst (Cleary et al., 2008), is required for behavioral withdrawal. Although RVM ON- and OFF-cell physiology has been well studied in males, few studies have recorded from RVM neurons in females. Building from these insights, our study indicates that in comparison with naive males the impact of estrogen fluctuations in intact, naïve females may be slight, altering sensitivity within the noxious stimulus range without significantly shifting nociceptive thresholds or associated cell activity. As stated above, a change in the stimulus-evoked activity of OFF-cells—but not ON-cells—is required for behavioral withdrawal. In our study the mechanical threshold (or the likeliness to withdraw at a given fiber force) was nearly identical for males and females, consistent with the lack of significant differences in cell-activity of either ON- or OFF-cells.

Although naïve males had shorter withdrawal latencies than females, the mechanical sensitivity and associated cell activity of male and female did not significantly differ after the induction of persistent pain. To assess mechanical sensitivity, we used vF fibers on rats of both sexes before and after unilateral injections of CFA into the hind paw. In contrast to thermal sensitivity, sex differences in pressure pain and mechanical sensitivity are more frequently reported (Bradshaw et al., 2000, Nicotra et al., 2014) but is still contested (Racine et al., 2012). After treatment with CFA, both female

and male rats developed significant unilateral hypersensitivity that was associated with changes in the evoked firing of ON- and OFF-cells in the RVM. Reports of sex differences in mechanical sensitivity past the acute phase of hypersensitivity are mixed, with some studies suggesting that females are more sensitive while others report no differences (Auh & Ro 2012, Cook & Moore 2006). Some of these inconsistencies may be attributable to experimental design factors such as strain, which particularly seems to impact the detection of sex differences (DeLeo & Rutkowski 2000, Grisel & Mogil 2000). Nonetheless, many studies that found differences between males and females assessed behavioral responses only, leaving open the question as to whether and how they may differ in the development of central sensitization. (Auh & Ro 2012, Carlson et al., 2007, Cook & Moore 2006, Jinks et al., 2004, Oliveras et al., 1991, Tonsfeldt et al., 2016).

The lightly anesthetized preparation we use in extracellular recording may alter the detection of behavioral sex differences, both before and after CFA-treatment. Descending GABAergic tone from vlPAG neurons that project to the RVM plays an important role in mediating hypersensitivity following CFA (Tonsfeldt et al., 2016), and manipulations that alter GABA release in the vlPAG and elicit hypoalgesia also alter responses of RVM ON- and OFF-cells. Tonsfeldt et al., (2016) found that tonic $GABA_A$ currents are unaffected by CFA in males, but are significantly attenuated in females, with a concomitant increase in GABA release in vlPAG. Therefore, it is possible that sex differences resulting from subtle changes in $GABA_A$ tone may be obscured under even light barbiturate anesthesia (such as under methohexital) which acts on $GABA_A$Rs. Indeed, the depth of anesthesia has been shown to moderate ON- and OFF-cell activity (Jinks et al., 2004, Oliveras et al., 1991), and paw withdrawal magnitudes to a noxious mechanical stimulus are consistently lower in awake behaving rats regardless of whether they are healthy controls, nerve-injured animals, or sham-operated controls (Carlson et al., 2007). However, the ability to detect injury-induced allodynia and

hyperalgesia under light anesthesia remained intact. Importantly, in Tonsfeldt et al., (2016) sex differences in $GABA_A$ signaling were associated with differences in morphine-induced antinociception, but not with behavioral differences in paw withdrawal latencies at baseline or following CFA. Specifically relevant to my studies is the consideration of whether anesthesia may mask behavioral sex differences that would otherwise be apparent in awake, behaving rodents. While relatively fewer studies of nociception have been performed in anesthetized females, behavioral comparisons between awake behaving males and females are similar to what I found using the same noxious stimuli (Auh & Ro 2012, Cook & Moore 2006). Nonetheless, there are reports of differential activation in relays for pain transmission and other pathways that influence the evoked firing of RVM neurons in response to noxious stimuli (Dickie et al., 2017, Herrero et al., 2000, McRoberts et al., 2007, Murphy et al., 2009, Nazarian et al., 2014), raising the possibility that inputs that contribute to sex differences in an awake subject could be diminished or obscured in even a lightly anesthetized rat.

Several studies have identified sexual dimorphism in relays that transmit ascending nociceptive information. Acute, somatic pain is initiated in the periphery, with activation of C- and Aδ-fibers, and one recent investigation found that C-fibers of male and female rats displayed differences in activity-dependent slowing, a reduction in the velocity of action potentials in response to repetitive stimuli that facilitates spinal summation (Dickie et al., 2017). These fibers synapse onto secondary neurons in the spinal dorsal horn, the majority of which have axons that cross the midline before ascending to supraspinal recipients of nociceptive input like the thalamus, PB, PAG, and RVM. Primary afferents in female rats also release more substance P (a pronociceptive polypeptide) in the spinal cord than males or gonadectomized females (Nazarian et al., 2014). Substance P is thought to contribute to "wind-up", an amplification of sensory transmission that involves sensitization of secondary neurons of the dorsal horn (Herrero

et al., 2000). Female sex appears to result in other mechanisms of amplification at the spinal level: NMDA-induced excitatory currents in the dorsal root ganglia of female rats are greater than currents measured in DRGs from males. Meanwhile, neurons in the spinoparabrachial pathway, an important source of ascending nociceptive information to the RVM, are more activated in males by a painful visceral stimulus than in females (Murphy et al., 2009), and are more suppressed by morphine.

Sex differences in supraspinal areas that project to RVM ON- and OFF-cells have been found. Painful stimuli can induce significant differences in functional activation in female rats relative to males. Wang et al., (Wang et al., 2009) recorded increased cerebral blood flow in response to painful colorectal distension in many areas of that may modulate RVM neuron activity, including the PB, insula and ACC. Both the PB and ACC have direct projections to the RVM, and in the case of PB neurons, can affect paw withdrawal latencies and alter associated ON- and OFF-cell activity (Roeder et al., 2016). Differences in activation after inflammation have been found in PAG neurons that project to the RVM (Loyd et al., 2007), while GABAergic signaling is altered by the induction of inflammatory pain in a sex-dependent manner (Tonsfeldt et al., 2016). The PAG (Morgan et al., 2008) and PB (Chen et al., 2017) both provide important efferent inputs onto pain-modulating neurons in the RVM, and can significantly alter evoked neuronal activity there, and induce either hypo- or hypersensitivity depending upon the intervention. In fact, we found that while injured rats showed no significant differences in behavioral or cell responses to either thermal or mechanical stimuli, naïve males had shorter latencies to withdraw when the 60g vF fiber was tested, although they were as likely to withdraw and thus had similar mechanical thresholds. Although there are activational differences in other areas related to pain transmission, including areas that provide critical inputs to pain modulating neurons, our data show that at the level of

the RVM, the output of descending control, males and females are largely similar, despite differences in behavioral sensitivity in the naïve state.

In conclusion, our results suggest that RVM pain modulating neurons are functionally similar in males and females, and undergo similar alterations in evoked activity following induction of persistent inflammatory pain. Taken together, these results suggest that although sex differences in the amplification of pain transmission have been reported at different peripheral and supraspinal sites, at the level of the RVM, the neuronal activity of males and females was not detectably different.

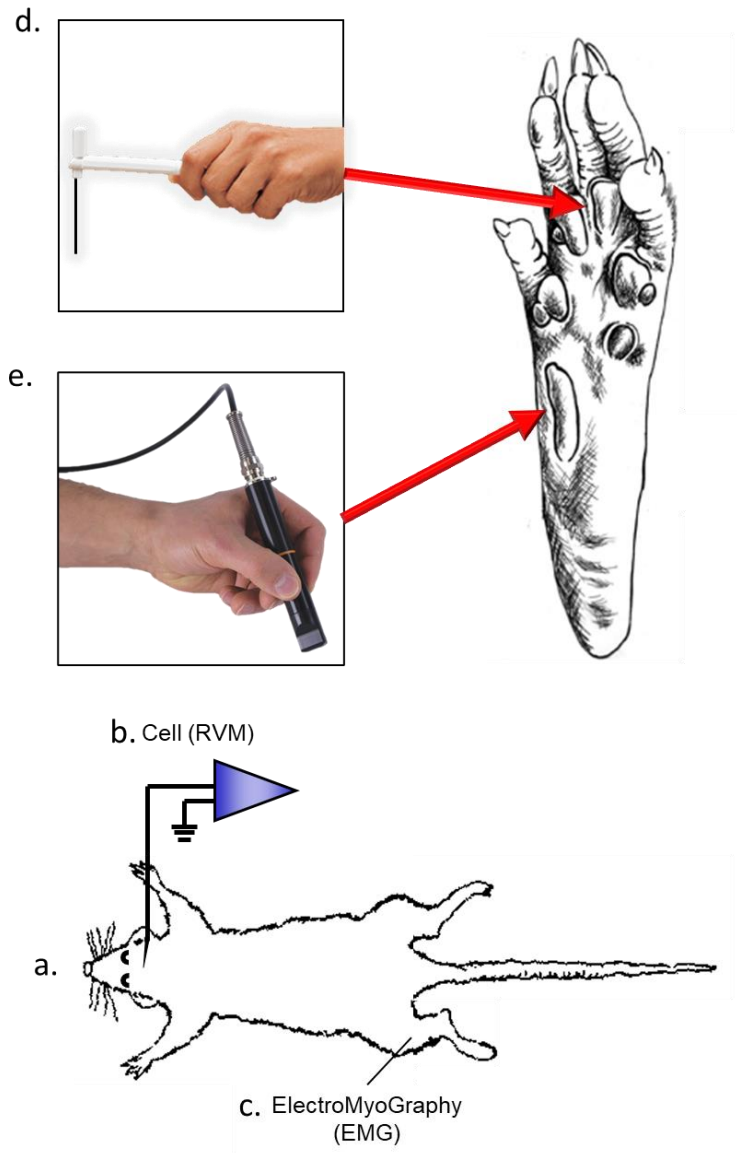


Figure 3: Experimental set-up
Once stabilized in the stereotaxic frame (A), recordings from RVM neurons were collected (B) while an EMG in each hindleg (C) allowed for concurrent behavioral measurement. Mechanical sensitivity was determined using vF fibers (D) applied to the webbing between the toes. Thermal sensitivity was assessed using a Peltier probe (E) applied to the plantar surface of the hind-paw.

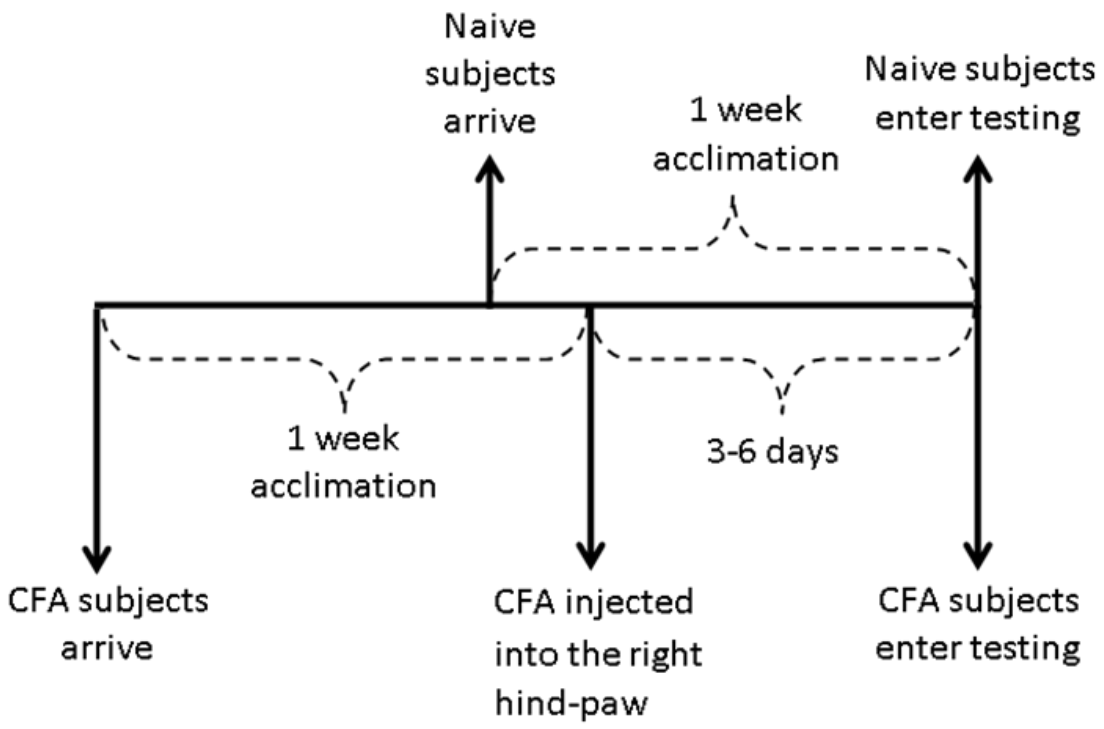


Figure 4: Experimental timeline
After arrival, but prior to handling and testing, all subjects were housed in the vivarium for one week to acclimate. Subjects in the naïve groups were then available for testing. For subjects in the CFA treated group, a 0.1ml of 1 mg/1 ml dose of CFA was injected subcutaneously in the right hind-paw under isoflurane-anesthesia in the days after acclimation. CFA-treated subjects were then returned to their home cages for 3-6 days, and then entered into testing sessions. For all subjects, testing involved one session that began with surgery to implant a catheter for continuous anesthesia followed by single-unit recording with concurrent behavioral testing.

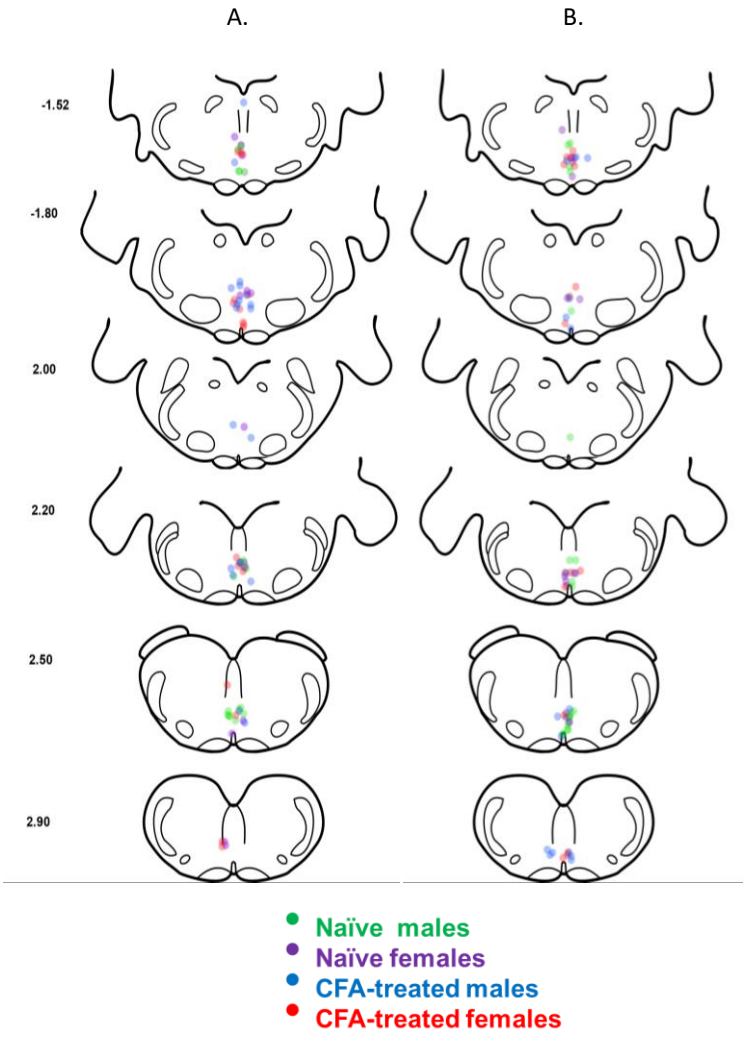


Figure 5: Location of recording sites within the RVM

A. ON-cells in uninjured and CFA-treated males and females were distributed between sections – 1.52 mm and 2.90 mm relative to the interaural line. The majority of cells were recorded between -1.52 mm and 2.50 mm.
B. OFF-cells in uninjured and CFA-treated males and females were distributed between sections – 1.52 mm and 2.90 mm relative to the interaural line. The majority of cells were recorded between -1.52 mm and 2.50 mm.

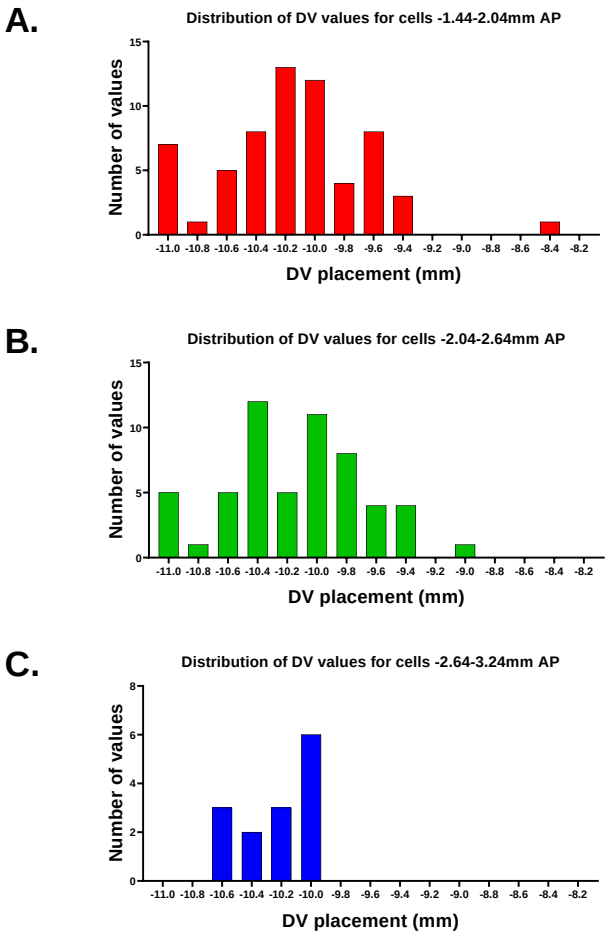


Figure 6: Distribution of recording sites within the RVM
Neuronal recordings placements were plotted according to their dorsoventral (DV) depth. For neurons found between -1.44mm and -2.04mm AP (A), the majority of recorded cells were found at a distance of -9.4-10.5 mm DV, while those found between -2.04mm and -2.64mm AP (B) were found at a distance of -9.4-10.6 mm DV. Relatively few neurons were recorded in the range of -2.64mm and -3.24mm AP (C), with the majority of these cells found at a distance of -10.0-10.6 mm DV.

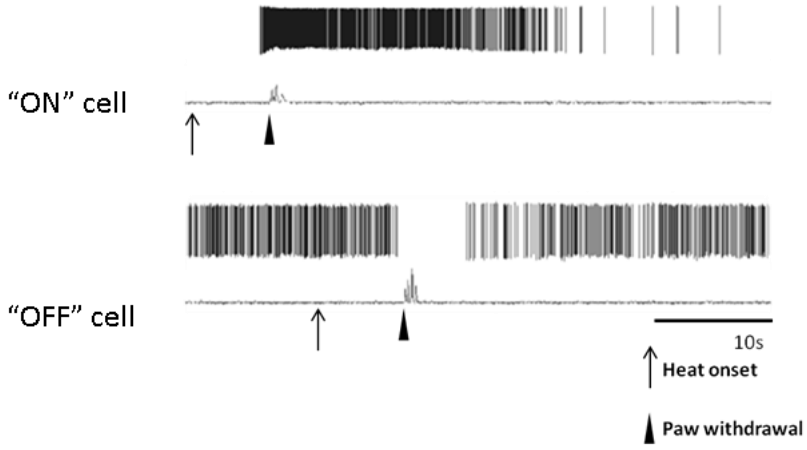


Figure 7: Temporal pattern of ON- and OFF-cell activity
Representative examples of the firing pattern of an ON- and an OFF-cell during withdrawal from a noxious thermal stimulus. EMG recording from the gastrocnemius muscle is used to determine the onset of withdrawal.

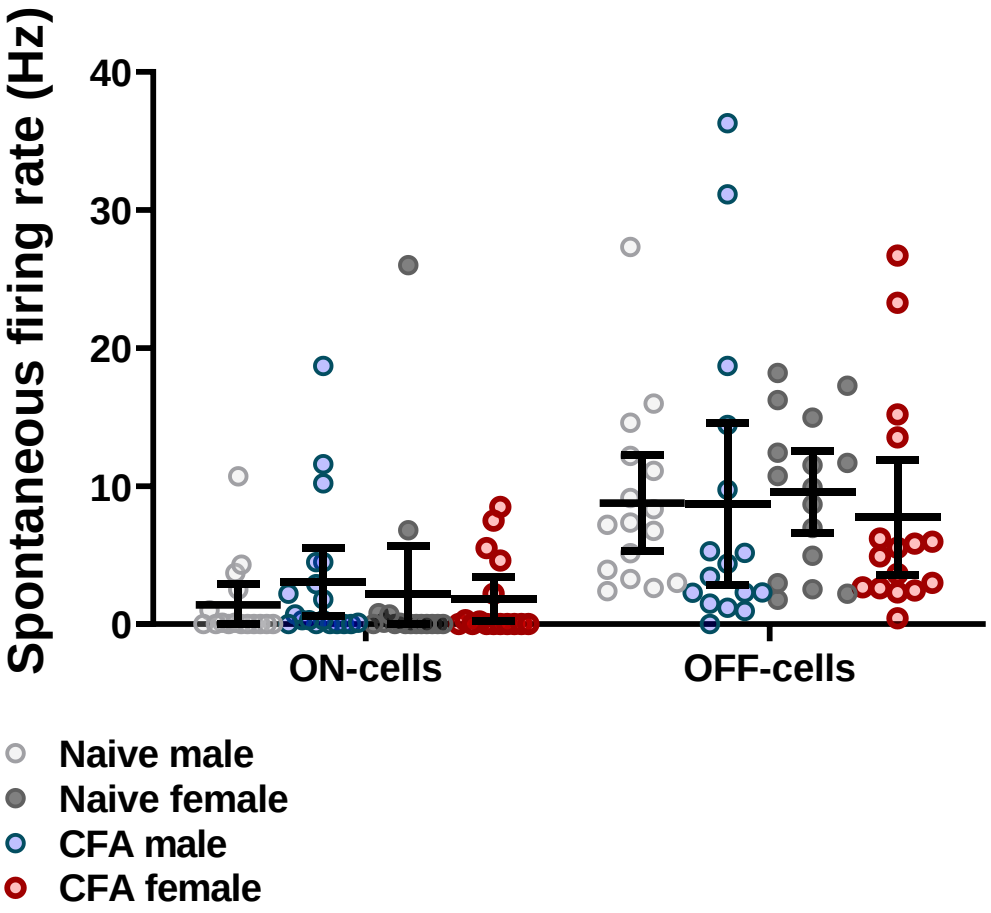


Figure 8: Spontaneous activity of ON- and OFF-cells
Ongoing firing rates of ON-cells (16-19 per group) and OFF-cells (n=15-16 per group) were not significantly affected by either sex or treatment with CFA. (two-way ANOVA: Sex: ON-cells, $F(1,62)=0.02699$, $p=0.87$; and OFF-cells, $F(1,60)=0.2442$, $p= 0.62$. CFA: ON-cells, $F(1,62)=0.3745$, $p=0.54$; and OFF-cells, $F(1,60)= 2.899$, $p = 0.094$). Reported as geometric mean and 95% CI.

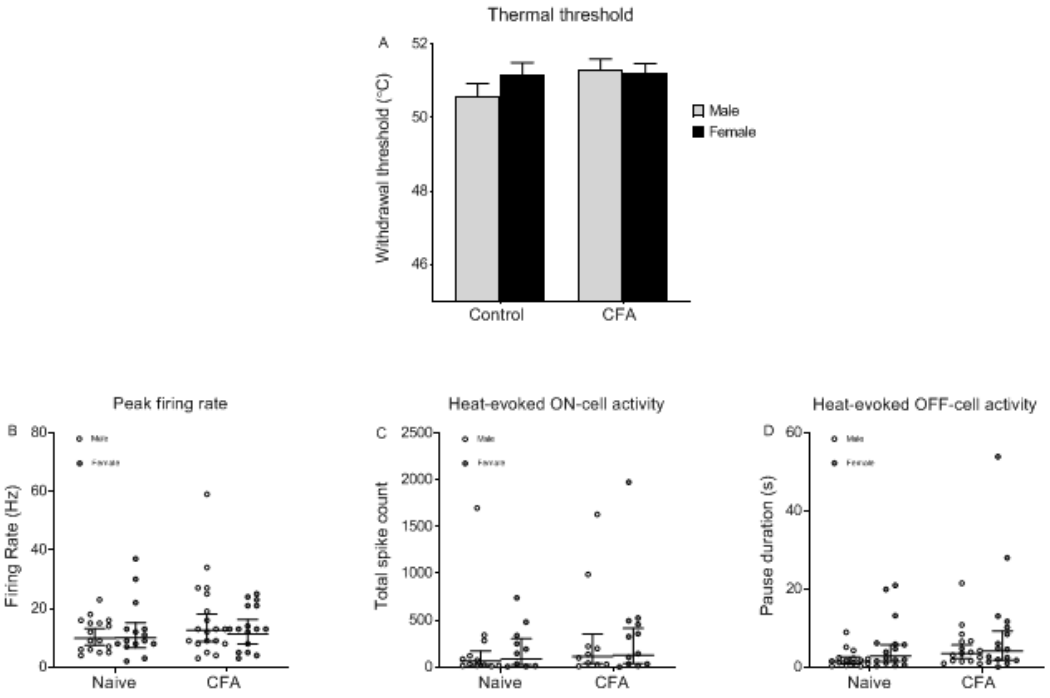


Figure 9: Thermal latencies and cell behavior before and after CFA-treatment

A. Thresholds for heat-evoked withdrawal in males (n=20-30 per group) and females (n=22-31 per group) were not significantly different females: $F(1,99)=0.6158$, $p=0.43$. There was no effect of CFA treatment on withdrawal latency: $F(1,102)=0.1.303$, $p=0.26$. Reported as mean and SEM.
B. The peak firing rates recorded around the ON-cell burst were not different between males and females, and between CFA-treated rats and uninjured controls. (two-way ANOVA, sex: $F(1,62)=1.55$, $p=0.22$; CFA: $F(1,62)=0.0801$, $p=0.78$, n=15-19 per group). Reported as geometric mean and 95% CI.
C. The total number of spikes in the ON-cell burst associated with the withdrawal to heat did not differ between male and female rats. Consistent with the absence of enhanced thermal sensitivity after CFA, the number of spikes did not significantly change relative to the uninjured controls. (two-way ANOVA, sex: $F(1,40)=0.85$, $p=0.36$; CFA: $F(1,40)=0.0099$, $p=0.9211$, n=10-12 per group). Reported as geometric mean and 95% CI.
D. The duration of the OFF-cell pause was not significantly different between males and females, or between CFA-treated rats and naïve controls. (two-way ANOVA, sex: $F(1,59)=2.63$, $p=0.11$; CFA: $F(1,59)=3.47$, $p=0.067$, n=15-16 per group). Reported as geometric mean and 95% CI.

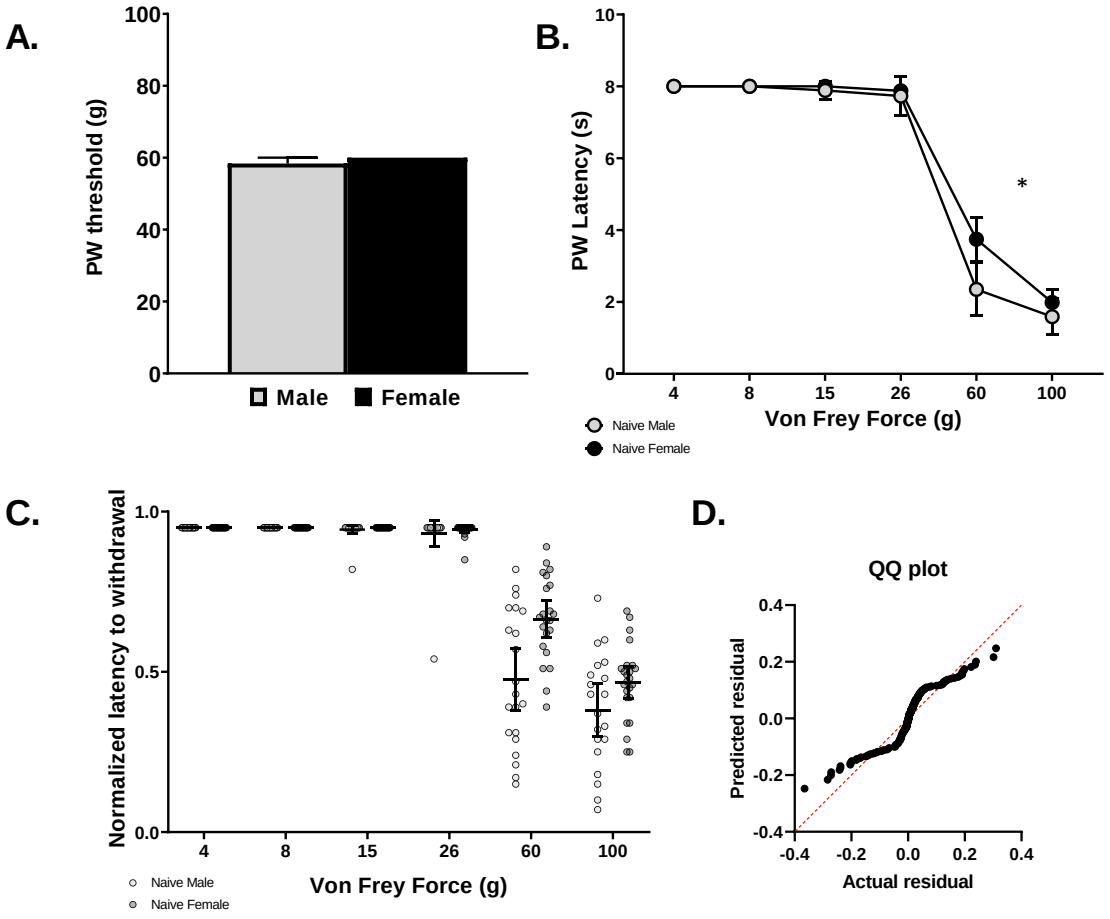


Figure 10: Mechanical sensitivity and behavioral latencies to withdraw in naïve rats

A. Mechanical withdrawal thresholds were assessed on both hind paws for male and female rats. Paw withdrawal thresholds did not significantly differ based on sex. (Mann-Whitney test: p=0.45, n=21-26 per group). Reported as mean and SEM.
B. The latency to paw withdrawal after a vF fiber was significantly shorter for males than for females: (Mixed-effects model (REML), Sex: F(1,43)=11.04, *p>0.05, n=20-25 per group). Reported as geometric mean and 95% CI.
C. Because withdrawal behavior is often skewed, latencies to withdrawal were log-transformed prior to analysis. Normalized latencies to withdrawal for each subject are represented.
D. QQ-plot of the log-transformed withdrawal latencies for all groups.

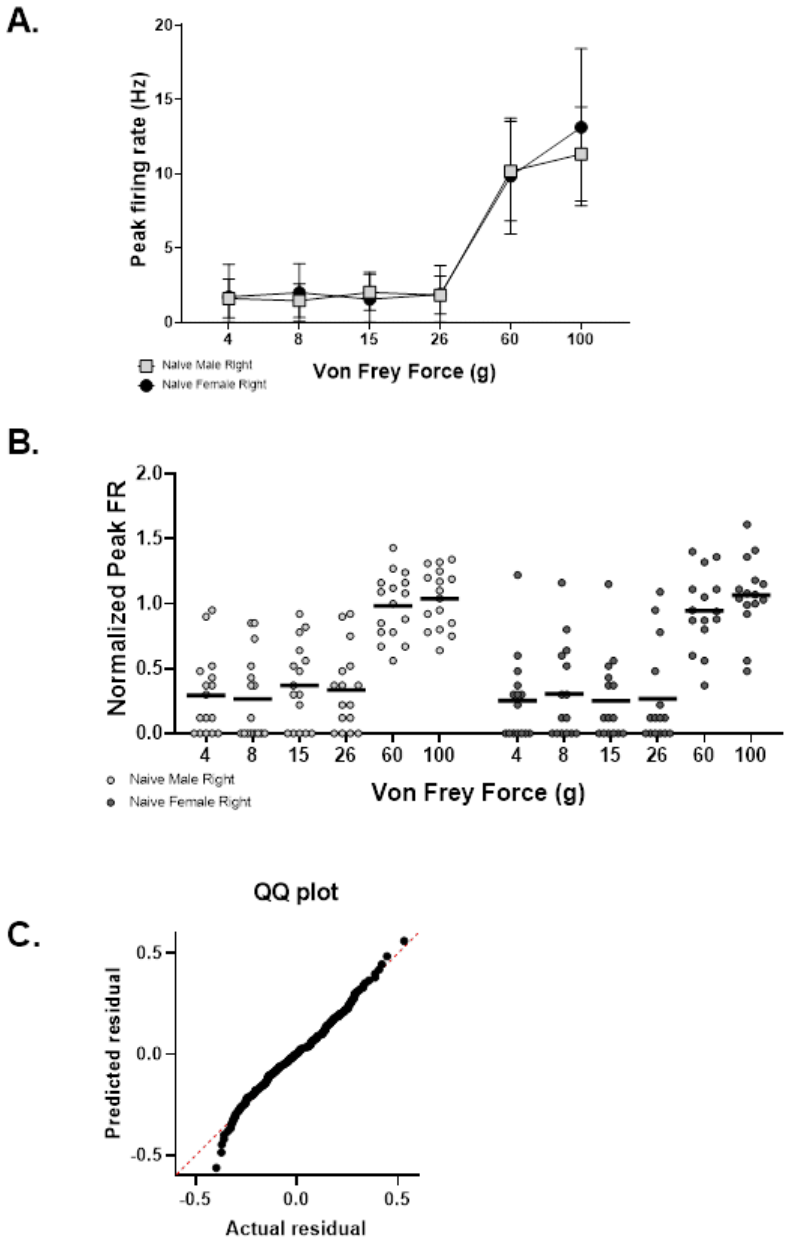


Figure 11: Peak firing rate of ON-cells in naïve males and females

A. There were no differences in the firing rates associated with withdrawal to fibers in the noxious range for male and female rats (two-way repeated measures ANOVA, Sex: $F(1,29)=0.15$, $p=0.70$, n=15-16 per group.) Reported as geometric mean and 95% CI.
B. Similar to withdrawal behavior, the cell activity data typically follows a non-normal distribution. Prior to analysis, all cell data was log-transformed to approximate a more normal distribution. Normalized peak firing rates for each individual data point are displayed.
C. QQ-plot of the normalized firing rate data shows that an approximately normal distribution is achieved by log-transformation.

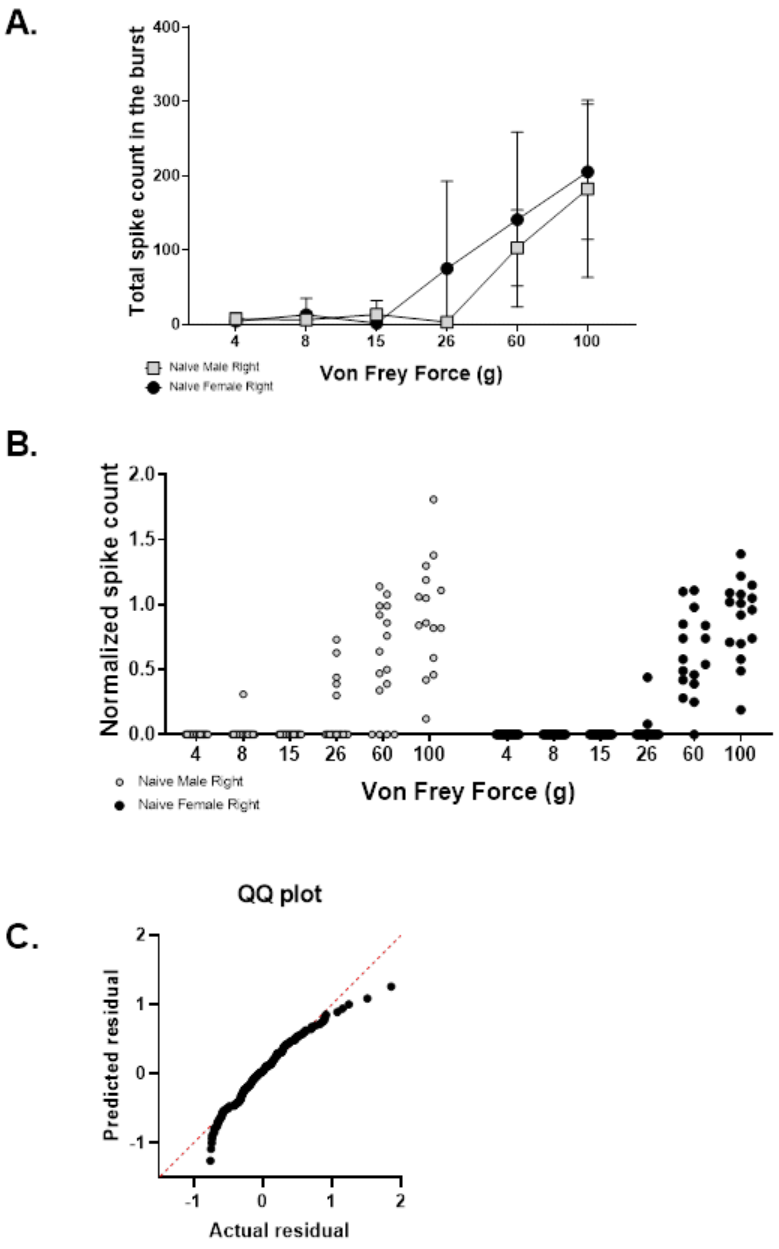


Figure 12: Evoked ON-cell activity in naive males and females

A. The total number of spikes counted in the ON-cell burst was also not different between the sexes. (two-way repeated measures ANOVA, Sex: $F(1,29)=0.14$, $p=0.71$, n=15-16 per group.) Reported as geometric mean and 95% CI.
B. The total spike counts were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.
C. Q-Q plot for the log-transformed data.

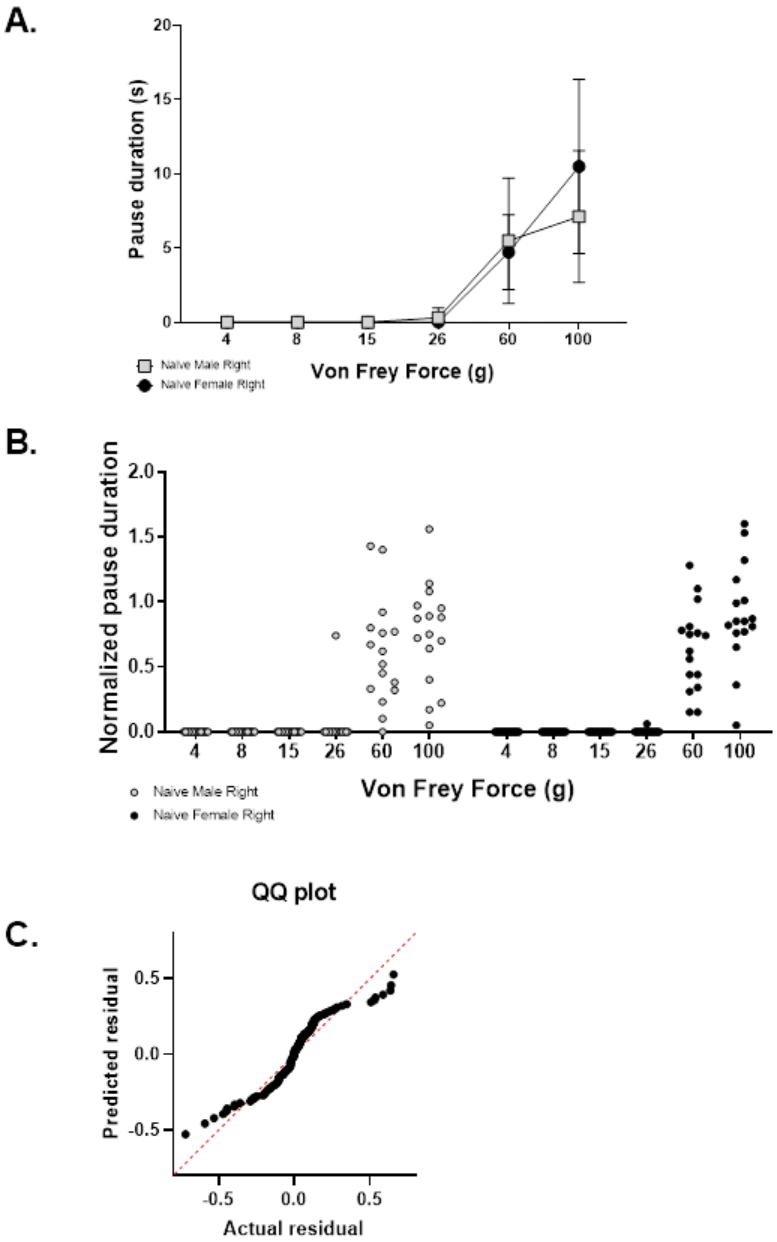


Figure 13: Evoked OFF-cell activity in naïve males and females

A. The duration of the OFF-cell pause also did not differ in males and females (Mixed-effects model (REML), Sex: F(1,30)=0.33, p=0.57, n=16 per group.) Reported as geometric mean and 95% CI.
B. The durations of the OFF-cell pause were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.
C. Q-Q plot for the log-transformed data.

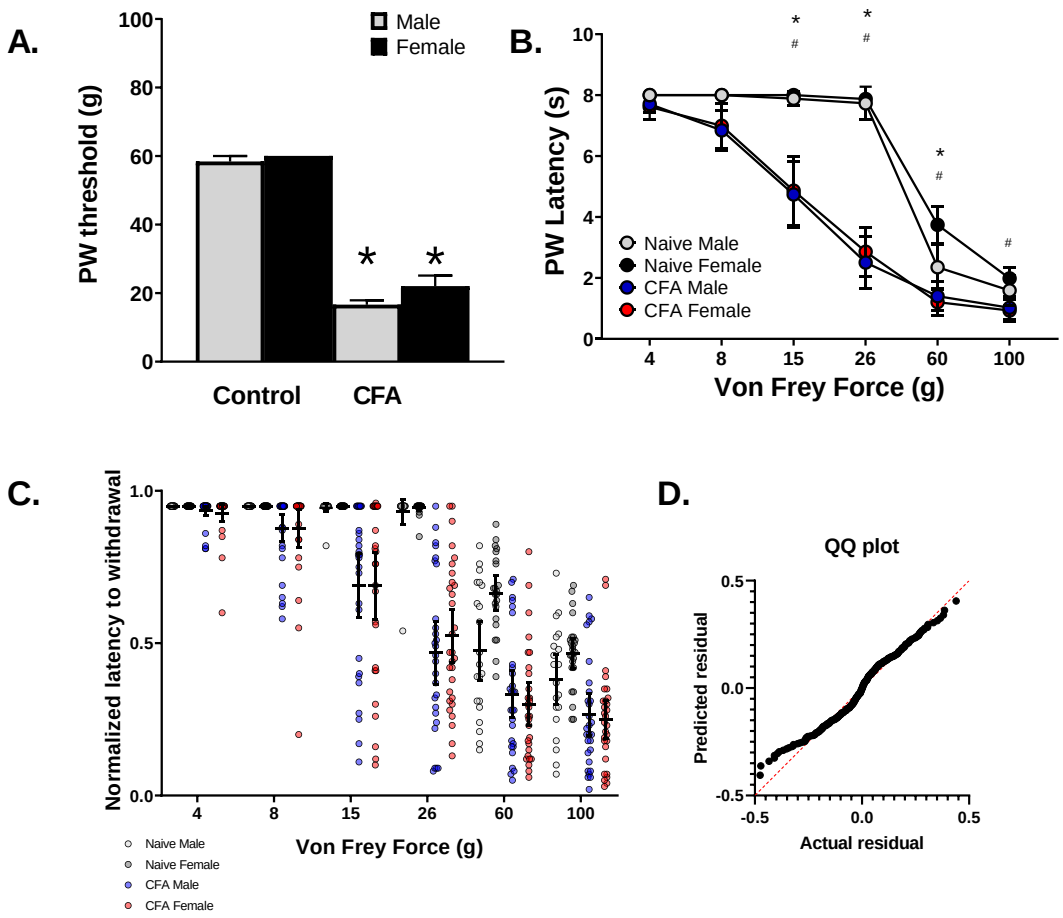


Figure 14: Unilateral mechanical hypersensitivity in CFA-treated male and female rats

A. Paw withdrawal thresholds in CFA-treated rats were significantly lowered for both males and females when compared with naïve controls, but did not differ based on sex (two-way ANOVA, with Sidak's multiple comparison's test, CFA: $F(1,103)=388.3$, *$p<0.0001$ when compared to same-sex control; Sex; $F(1,103)=3.027$, $p=0.084$, n=21-31 per group). Reported as mean and SEM.
B. The latency to the paw withdrawal was also shorter in CFA-treated rats, but did not differ between males and females in the CFA-treated groups (three-way ANOVA, CFA: $F(1,102)=105.8$, $p<0.0001$; Sex: $F(1,102)=1.472$, $p=0.23$, n=20-31 per group). Reported as geometric mean and 95% CI.
C. Normalized latencies to withdrawal for each subject are represented.
D. QQ-plot of the log-transformed withdrawal latencies for all groups. Latencies to withdrawal were log-transformed prior to analysis.

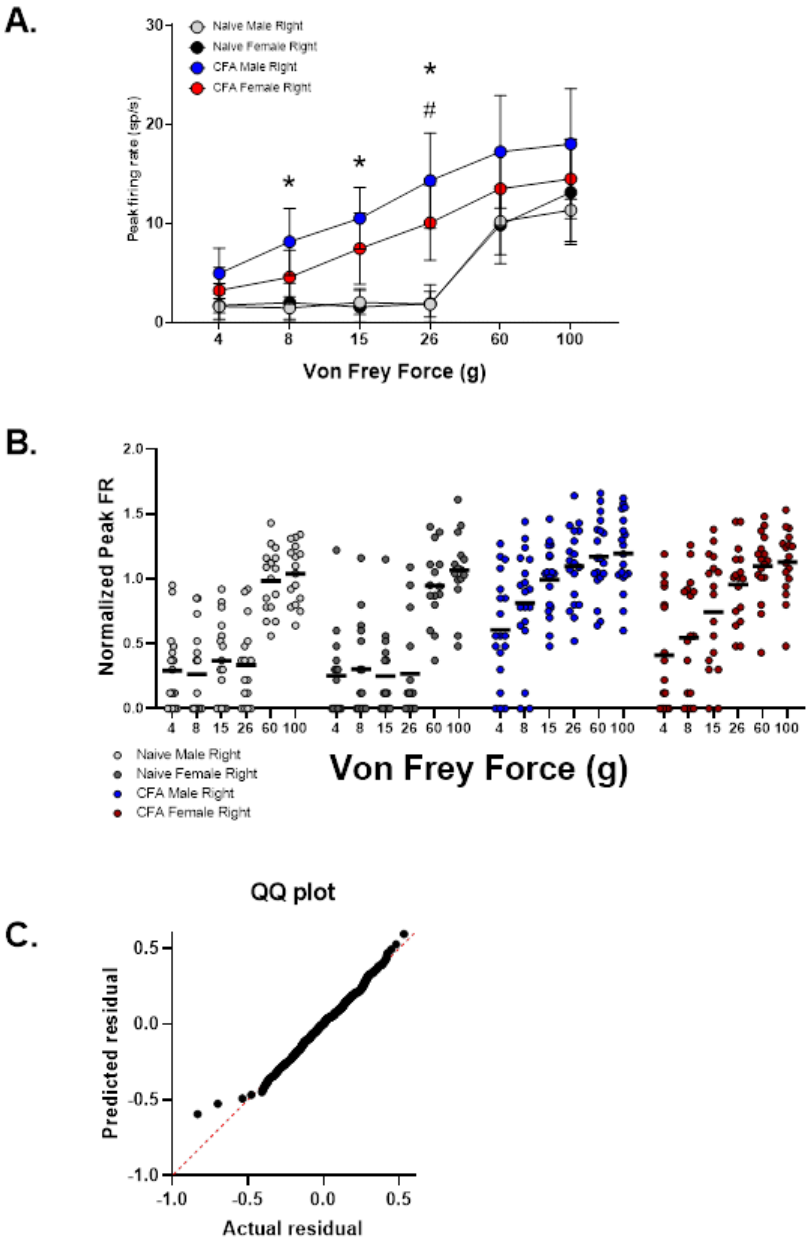


Figure 15: Peak firing rates of ON-cells after CFA-treatment

A. The peak firing rate of ON-cells associated with the withdrawal was significantly higher in rats treated with CFA when compared to controls (three-way ANOVA, with post-hoc Tukey's multiple comparison's test: $F(1,63)=31.76$, $p<0.0001$, n=15-19 per group. Male CFA-treated compared with naïve: *$p< 0.05$. Female CFA-treated compared with naïve: # $p<0.05$). However, there was no effect of sex : $F(1,63)=2.33$, $p=0.13$. Reported as geometric mean and 95% CI.
B. The total spike counts were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.
C. Q-Q plot for the log-transformed data.

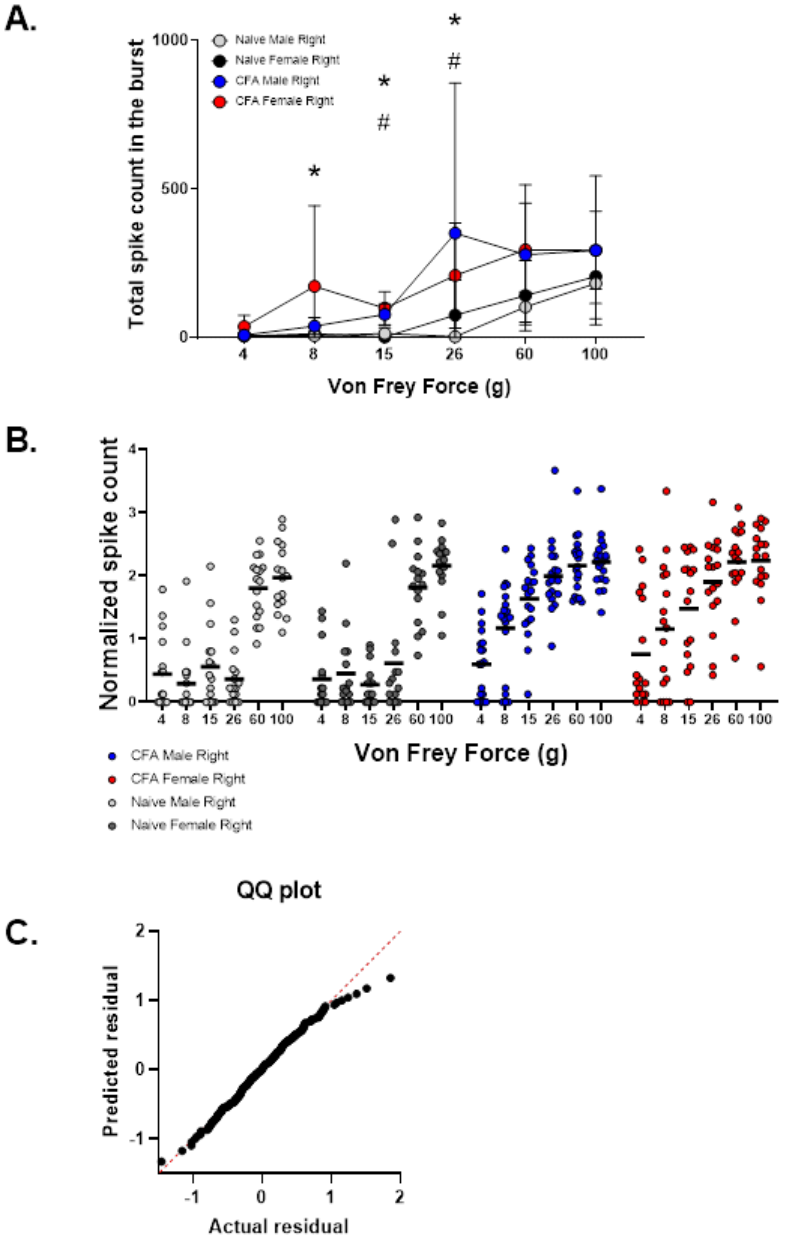


Figure 16: Evoked activity of ON-cells after CFA-treatment

A. The total number of spikes in the ON-cell burst was significantly higher after treatment with CFA (three-way ANOVA, with post-hoc Tukey's multiple comparison's test. Male CFA-treated compared with naïve:*p<0.05. Female CFA-treated compared with naïve: # p<0.05. F(1,63)=43.96, p<0.0001, n=15-19 per group), but there was no effect of sex: F(1,63)=0.029, P=0.86. Reported as geometric mean and 95% CI.
B. The total spike counts were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.
C. Q-Q plot for the log-transformed data.

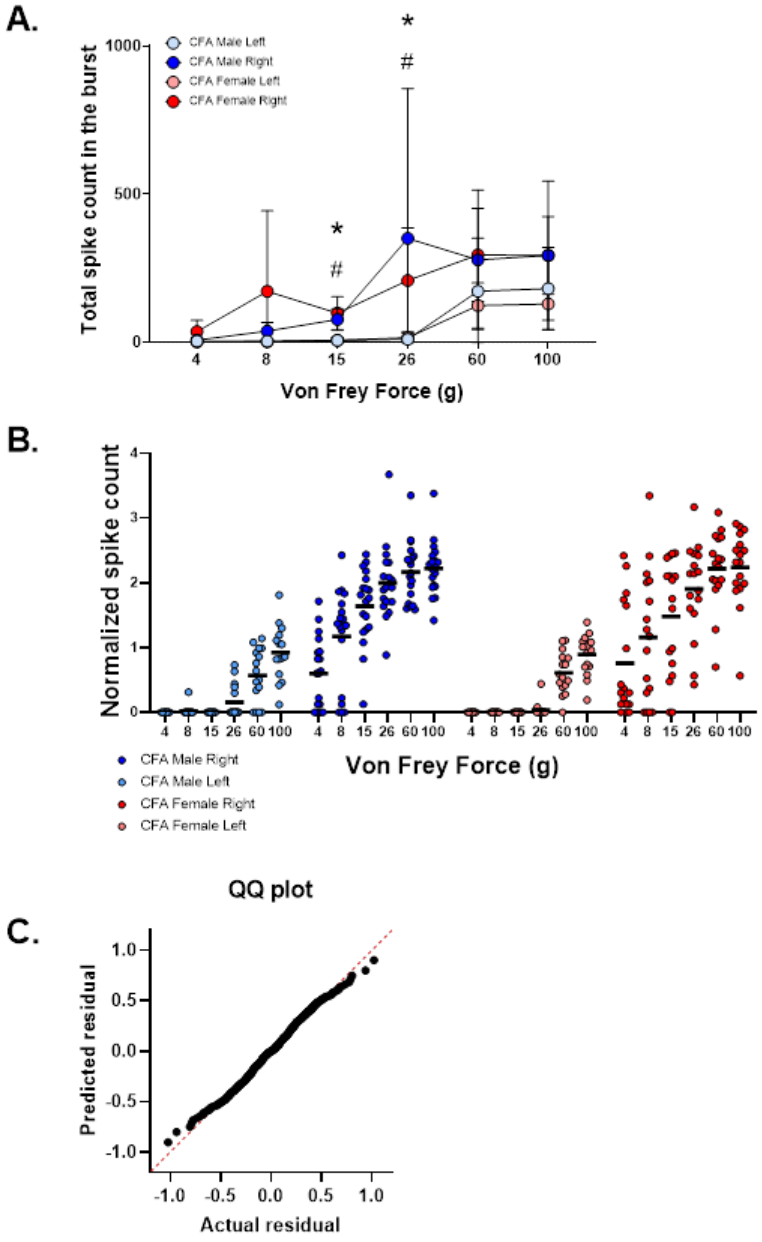


Figure 17: Evoked activity of ON-cells after CFA-treatment

A. The total number of spikes in the ON-cell burst was significantly higher when the injected paw was tested than when the contralateral untreated paw was tested (three-way ANOVA, with post-hoc Tukey's multiple comparison's test. $F(1,34)=88.22$, $p<0.001$, n=17-19 per group. Male treated versus untreated paw:*$p<0.05$. Female treated versus untreated paw: # $p<0.05$). There was no effect of sex: $F(1,34)=0.37$, $p=0.55$. Reported as geometric mean and 95% CI.
B. The total spike counts were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.
C. Q-Q plot for the log-transformed data.

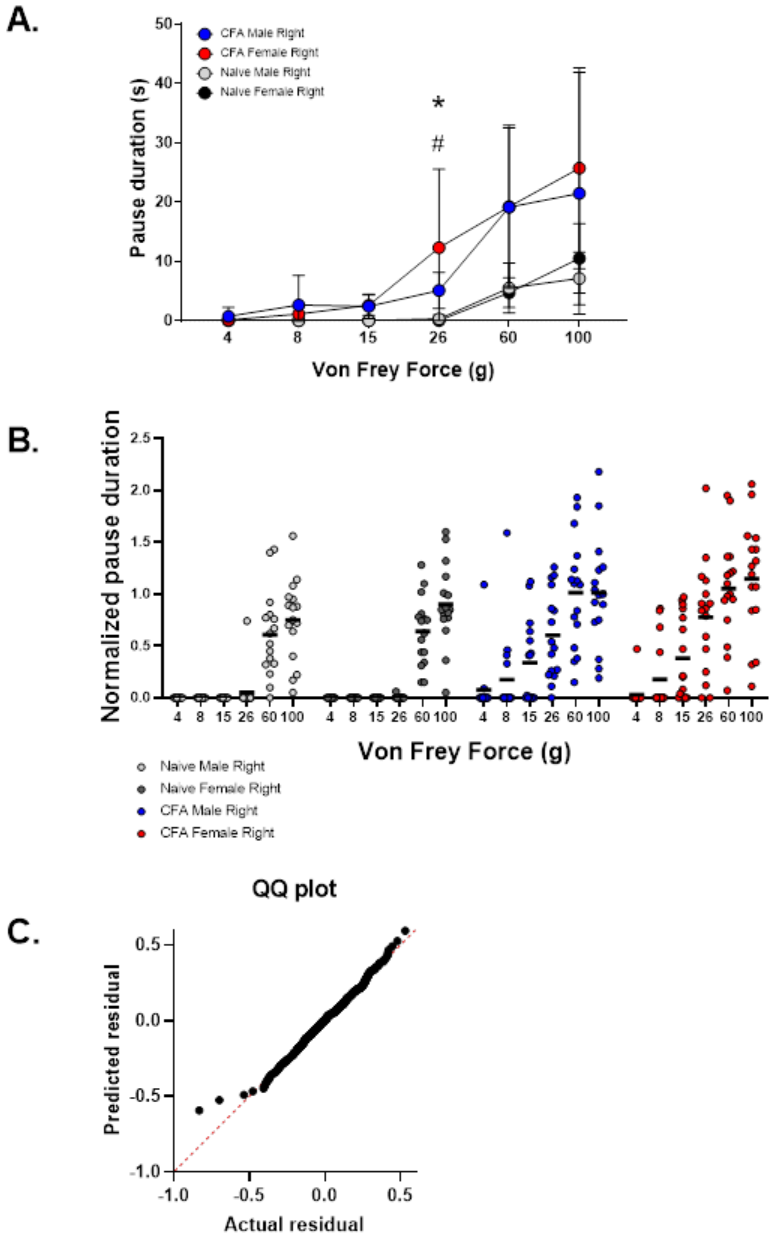


Figure 18: Evoked activity of OFF-cells after CFA-treatment

A. The duration of the OFF-cell pause was significantly longer when the injected paw of CFA-treated animals was tested compared to the same paw in naive animals (mixed-effects model (REML), $F(1,60)=31.43$, $p<0.0001$) but did not differ between males and females ($F(1, 60)=0.57$, $p=0.45$. N=16 per sex). Reported as geometric mean and 95% CI.
B. The distributions of the OFF-cell pause duration were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.
C. Q-Q plot for the log-transformed data.

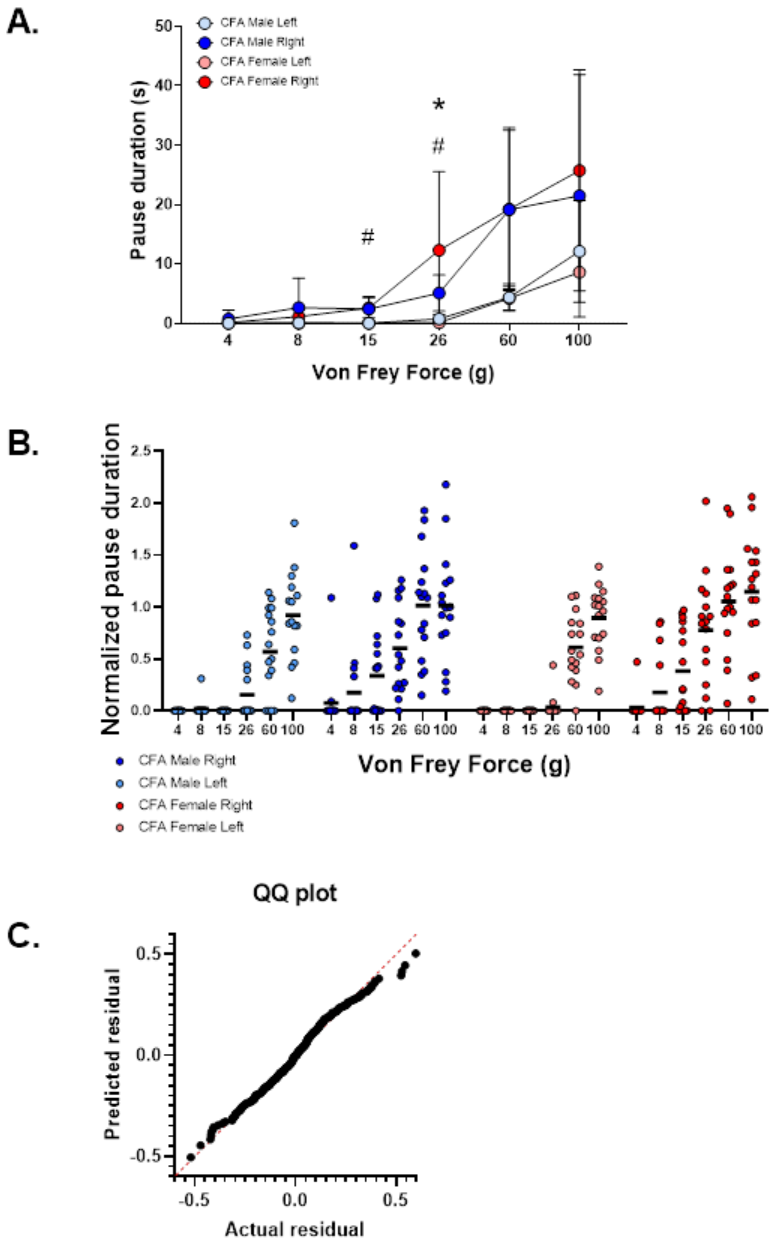


Figure 19: Evoked activity of OFF-cells after CFA-treatment

A. The duration of the OFF-cell pause was significantly higher when the injected paw was tested than when the contralateral untreated paw was tested in CFA-treated animals (mixed-effects model (REML), $F(1,30)=40.25$, $p<0.0001$) but did not differ between males and females ($F(1,149)=0.081$, $p = 0.78$. N= 16 per sex). Reported as geometric mean and 95% CI.

B. The distributions of the OFF-cell pause duration were normalized with a log-transformation prior to analysis. Individual data for each subject are displayed.

C. Q-Q plot for the log-transformed data.

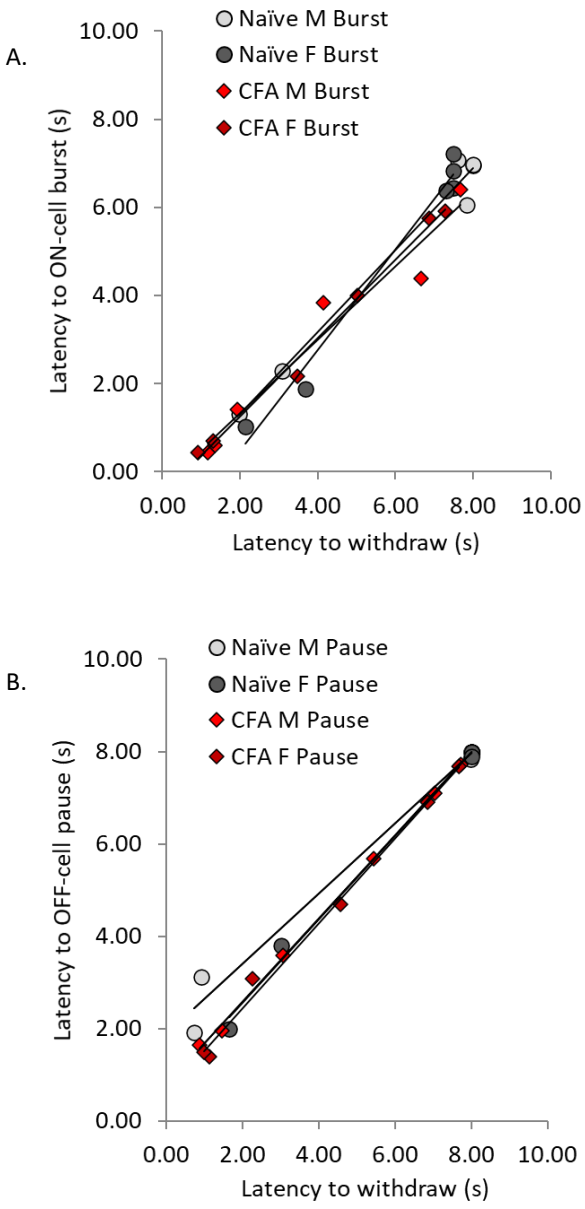


Figure 20: Relation between latency to behavioral withdrawal and latency to change in cell activity

A. The latency from the onset of the stimulus to the moment of behavioral withdrawal, and the latency to the beginning of the ON-cell burst were plotted for each group.
B. Likewise, the latency from the onset of the stimulus to the moment of behavioral withdrawal, and the latency to the beginning of the OFF-cell pause were plotted for each group.

CHAPTER 3

DISCUSSION

3.1. KEY FINDINGS

- Thermal thresholds in naïve male and female rats were similar, as were spontaneous and evoked responses of ON- and OFF-cells.
- Mechanical withdrawal thresholds in naïve male and female rats were similar, and associated with comparable ON- and OFF-cell evoked responses. For males, the latency to withdraw was shorter.
- Thermal sensitivity and heat-evoked neuronal responses were unchanged by persistent inflammation of the hind-paw, replicating earlier findings in males, and extending them to females.
- Male and female rats displayed similar levels of mechanical hypersensitivity on the treated paw. Evoked activity of ON- and OFF-cells was similar as well. This again replicates earlier findings in males that mechanical hypersensitivity after CFA alters evoked but not ongoing RVM neuronal activity, and extends these insights to females.

3.2. OVERVIEW

In this thesis, I assessed the hypothesis that sex differences in the physiology of pain-modulating neurons of the RVM exist, and that these neuronal differences are associated with differences in pain behaviors under basal conditions and in a model of persistent inflammatory pain. Using naive rats of both sexes, I found, instead, that the responses of ON- and OFF-cells in the RVM were similar between males and female for both noxious thermal and mechanical stimuli. Similarly, after the induction of persistent inflammatory pain, both sexes developed comparable levels of mechanical hypersensitivity, and associated ON- and OFF-cell responses were not significantly different between them.

These data argue that although differences in the pathways that provide nociceptive input to pain-modulating RVM neurons have been identified, the output of the pain-modulating circuitry, measured at the level of the RVM, is comparable in males and females. Thus, while RVM inputs have the potential to be differentially engaged by sex-linked physiological, behavioral and social contingencies, the same essential components exist in both sexes, and are similarly engaged by a local inflammatory challenge.

3.3. SEX DIFFERENCES IN INPUTS TO RVM DO NOT RESULT IN SEX-LINKED DIFFERENCES IN OUTPUT IN THE BASAL STATE

Interest in understanding the neural substrates of observed sex differences in clinical pain prevalence has led to the identification of sexual dimorphism in relays that transmit nociceptive information to the RVM. Activational and pharmacological differences between males and females have been identified in both ascending and descending pain pathways. These include differences in the activation of peripheral sensory neurons (Dickie et al., 2017, Nazarian et al., 2014, Ross et al., 2018), of neurons in the parabrachial nucleus, a key relay for nociceptive information to the RVM (Chen & Heinricher 2019, Chen et al., 2017, Murphy et al., 2009), and in GABA function in PAG (Loyd et al., 2008, Tonsfeldt et al., 2016). Despite this, there were no behavioral differences between males and females in my study, and the responses of RVM neurons in females were nearly identical to those of males with both thermal and mechanical stimuli. Because of the close relationship between RVM neuronal responses and behavioral output, this parallel similarity is coherent, and adds to a body of literature questioning a fundamental sex difference in basal sensitivity in rodents and humans.

3.4. RVM OUTPUTS ARE SIMILAR BETWEEN IN MALES AND FEMALES WITH PERSISTENT INFLAMMATORY PAIN

In chapter 2 I showed that the changes in activity and associated RVM neuronal responses in persistent inflammation were also similar between the sexes. Persistent inflammation from agents like CFA induces mechanical allodynia that is the result of sensitization of primary afferents as well as central sensitization, defined as "an amplification of neuronal signaling within the CNS that elicits pain hypersensitivity" (Woolf 2011), and is related to changes in many different sites (Woolf 2011), including the RVM (Robinson et al., 2002). Behavioral hypersensitivity co-occurs with molecular changes in RVM neurons that appear over the course of the first few weeks. Some modifications, such as an increase in phosphorylated AMPA receptors in RVM neurons (Guan et al., 2004) are maximal within the first few days and return to baseline levels before behavioral sensitivity resolves. Other alterations, affecting the expression and function of the neurokinin-1 receptor, trkB and opioid receptors (Guan et al., 2004, Guan et al., 2003b, Guan et al., 2002, Guo et al., 2006, Hurley & Hammond 2000, LaGraize et al., 2010, Ren & Dubner 2002, Schepers et al., 2008) persist far longer. Collectively, these changes contribute to a state characterized by normal spontaneous but altered reflex-related activity of ON- and OFF-cells, and lowered mechanical thresholds (Cleary & Heinricher 2013a). In many cases, this research has also been built upon studies that exclusively used male subjects, and so it remains to be seen whether males and females are similar at the molecular level.

Nonetheless, the responses of ON- and OFF-cells in males and females remained very similar even after the induction of persistent inflammation. This suggests that although there may be different molecular, cellular or non-neuronal mechanisms between the sexes that change in response to the induction of persistent inflammation,

males and females are able to use these mechanisms to achieve a very similar output at the level of the RVM. In fact, even in cases where the behavioral output of males and females is identical, the biological substrates of that output may nonetheless differ. A recent example of this was shown by Sorge et al., (2015) who found that male and female mice with a spared nerve injury (a rodent model of persistent pain in which the sciatic nerve is partially severed in a way that permits behavioral testing but induces hypersensitivity) recruit entirely different immune pathways during the development of mechanical allodynia. This was despite similar levels hypersensitivity developing in both sexes. Furthermore, treatments targeting the specific immune cells recruited by males after spared nerve injury were able to reverse allodynia only in males, leading Sorge et al., (2015) to conclude that males "should not be used as proxies for females in pain research." Moving beyond the mindset that important insights into the interactions of sex and pain processing start with the observation of behavioral differences in sensitivity will benefit future investigations into the mechanisms underlying the burden of clinical pain in women.

3.5. IMPLICATIONS FOR THE STUDY OF PAIN IN WOMEN AND MEN

Some variant of the same idea begins most publications on the subject of sex differences in pain research: "While sex differences in pain reporting are frequently observed, the reasons underlying these differences remain unclear". It is acknowledged within the clinical setting that women report more experiences of pain (de Mos et al., 2007, Dominguez et al., 2009, King et al., 2009, Leow et al., 2018, Ruau et al., 2012, Solheim et al., 2017, Tighe et al., 2015) and are more likely to have a diagnosis of chronic pain. Yet, determining the potential mechanisms for these differences has been hindered by theoretical and methodological issues in the study of sex differences generally, and specifically in pain research.

Interconnecting variables, such as demographics, diagnostic criteria and co-morbidities, have complicated assessing the biological substrates of sex differences in clinical pain. Eliot and Richardson (2016) identified causal dimensions that contribute to observed sex disparities as: psychological and sociological influences that can drive differences in reported pain that may not necessarily reflect differences in sensation, such as gender role and Social Learning Theory (Garofalo et al., 2006), dimensions correlated with sex, but not determined by it (such as muscle-to-fat ratio); and dimensions related directly to sex, such as sex chromosome complement (Gioiosa et al., 2008) or influences arising from direct interactions between gonadal hormones and relays in pain transmission (Tashiro et al., 2009). Explanations pulled from one dimension can sometimes exclude explanations pulled from another. For example, a recent study determined that men who adhered to a masculine gender role were more likely to volunteer for pain threshold studies in healthy subjects (Mattos Feijo et al., 2018). Since identification with masculine gender roles is correlated with higher pain thresholds, Mattos Feijo et al., (2018) suggest that this phenomenon could potentially account for the generally small differences between the pain thresholds of healthy men and women. However, this socio-cultural explanation raises an additional question: do differences in basal sensitivity, a popular hypothesis for the sex-based disparity in clinical pain prevalence, actually exist.

Certain basic assumptions, such as the relation between basal sensitivity and the likelihood of developing of chronic pain, continue to shape the study of sex differences in pain, even after the validity of these relations has been questioned by studies that do not assess sex as a variable of interest (Nielsen et al., 2009). When Mogil et al., (2000) reviewed sex differences in basal thermal sensitivity in rodents, they were able to find studies that fit every possible pattern of relation between male and female nociceptive sensitivity. They proposed several explanations for the lack of consensus concerning

sex differences including the possibility that the failure to report estrus phase, and random testing across the cycle, could account for missed detection of sex differences. They also acknowledged that identifying quantitative differences in basal sensitivity may be of limited value. The hypothesis that differences in pain sensitivity are a driver for the difference in chronic pain *prevalence* between men and women, however, was still being debated in later publications (Mogil 2012), although the literature supporting sex differences in pain sensitivity remains as equivocal as in the year 2000 (Melchior et al., 2016, Racine et al., 2012).

In fact, the sex difference in the prevalence of pain disorders is rarely assessed as a variable in experimental pain studies, which frequently exclude subjects who do not develop behavioral hypersensitivity. One criticism of experimental studies of pain is the difficulty of modeling the varied pain phenotypes that are present in clinical populations (Yezierski & Hansson 2018). Not everyone who suffers a peripheral nerve-injury develops neuropathic pain, yet rodent models investigating neuropathic pain (Kabli & Cahill 2007, Lynch et al., 2004, Malan et al., 2002) typically exclude subjects from analysis that do not develop mechanical hypersensitivity and/or cold allodynia. Although these forms of hypersensitivity replicate common symptoms of neuropathic pain syndromes, the exclusion of subjects that *do not* exhibit these symptoms could hinder the identification of the mechanisms that are specific to the development of persistent hypersensitivity and central sensitization, in contrast to mechanisms that may constitute a response to the *injury* in the absence of persistent behavioral hypersensitivity.

The exclusion of pain-free phenotypes in models of persistent pain creates several problems with regard to the study of sex differences. First, by excluding subjects who do not develop behavioral measures of hypersensitivity, differences in the proportion of males and females who go on to develop behavioral sensitivity may be missed. Rodent strain has been shown to affect the temporal pattern of the development

of mechanical allodynia after nerve injury as well as reports of sex differences in the magnitude of mechanical hypersensitivity (DeLeo & Rutkowski 2000). But strain may also have an effect on the proportion of males versus females that develop mechanical hypersensitivity, and this may be more clinically relevant than differences in the magnitude of hypersensitivity. Nevertheless, rodent studies are often also very poor at *reproducing* sex differences in chronic pain prevalence. For example, using the reserpine model of fibromyalgia, Hernandez-Leon et al., (2018) report an effect of estradiol on the development of behavioral hypersensitivity after reserpine administration. Yet, they did not find a significant difference between males and females. Since they only report differences in sensitivity, but not differences in the proportion of rats that developed hypersensitivity, it is difficult to assess whether the lack of apparent sex differences was due to confounding variables that hinder the detection of differences (as the effect of estrus cycle phase has been suggested to do) or to insufficiencies in the model of persistent pain that are sex-independent. Given that the ratio of female to male patients with fibromyalgia pain is quite high, it is not clear whether the reserpine model of fibromyalgia is inducing behavioral hypersensitivity by the same mechanisms in rodents as in human subjects.

Rodent studies allow for investigations into the mechanisms of pain transmission and modulation that are impossible in human subjects but are often poor at recreating the differences in prevalence and sensitivity seen in the clinical literature. This may be partly driven by the fact that some of the relevant mechanisms may be mediated by "top-down" influences that are difficult to account for in non-human subjects (e.g., pain-catastrophizing, defined as anxiety and fear of felt or anticipated pain (Lackner & Gurtman 2004)). Although my study demonstrated that males and females use common pain-modulating components at the level of the RVM, different social, psychological and environmental cues could result in different engagement of inputs from higher centers.

Studies designed to address disparities in chronic pain prevalence and care should not be predicated on the assumption that understanding why there are so many women with chronic pain is best answered by direct comparison with men. Rather, to gain a better understanding of the course of persistent pain within the female body, studies simply need to be performed with female subjects, regardless of whether they produce results that differ from those seen in males.

3.6. TECHNICAL CONSIDERATIONS

Anesthesia

The extracellular recording techniques in these experiments are by their nature invasive and are greatly facilitated by the use of light anesthesia. Yet, a possible concern with these experiments is the potential influence of anesthesia on behavior and RVM physiology.

One common criticism of the use of anesthesia in studies of behavioral nociception is the potential to blunt or mask nocifensive behaviors. In males, the use of the same surgical and anesthetic preparation to study behavior and cell responses is well established (Barbaro et al., 1989, Carlson et al., 2007, Cleary & Heinricher 2013a, Cleary et al., 2008, Heinricher & Fields 2013, Heinricher & Neubert 2004). Comparable stimulus intensities to those used in awake behaving animals can be employed with this preparation (Carlson et al., 2007, Cleary & Heinricher 2013a), and pharmacologic manipulations result in similar behavioral outcomes (Clarke et al., 1994, Oliveras et al., 1990). Additionally, RVM neurons identified in unanesthetized animals (Clarke et al., 1994, Hellman & Mason 2012, Leung & Mason 1999, McGaraughty & Reinis 1993, McGaraughty et al., 1995, Oliveras et al., 1989) have similar response dynamics to those recording in anesthetized animals, and respond proportionally to the intensity of stimulation (Oliveras et al., 1989). Although there may be quantitative differences

between the neuronal responses in lightly anesthetized and awake rats, anesthesia is likely to alter both ON- and OFF-cell responses, but not alter the direction of cell changes in response to nociceptive transmission, or their influence on behavioral outputs (Jinks et al., 2007, Leung & Mason 1999).

Specifically relevant to my studies is the question of whether the use of anesthesia may mask behavioral sex differences that would otherwise be apparent in awake behaving rodents. While relatively fewer studies of nociception have been performed in anesthetized females, behavioral comparisons in awake behaving rats using the same noxious stimuli I use are similar to what I found (Grisel & Mogil 2000). This reinforces the insight from studies with male subjects that although anesthesia may dampen the nocifensive reflex, it is unlikely to reverse behavioral responses. Nonetheless, since most studies that have illuminated the effects of anesthesia on nociceptive behaviors were performed in males exclusively, the role of anesthesia should be further explored.

3.7. FUTURE DIRECTIONS

3.7.1. Sex differences in opioid potency

The response to treatment with opioid drugs differs between men and women both in the clinical populations (Fillingim & Ness 2000, Gordon et al., 1993, Mogil & Bailey 2010, Niesters et al., 2010) and in preclinical research with human subjects. In humans, MOR and mixed-opioid agonists are often seen to be more potent in women than in men for experimental pain. For example, although women frequently report the same (Frot et al., 2004) or greater (Cepeda & Carr, 2003) postoperative pain, women often self-administer lower doses of opioid agonists (Chia et al., 2002) during patient-controlled analgesia. Nonetheless the magnitude of the pain intensity (experimental or clinically observed) may significantly influence whether there is a sex difference in opioid

potency, and how great that difference is. In their prospective cohort study, Cepeda and Carr (2003) found that women reported both more intense pain and required more morphine to achieve a similar degree of analgesia to that seen in males. This hints at the possibility that opioid-induced analgesia follows a very different dose-response curve in men and women, depending upon the level of pain it is used to treat. At the same time, women are more at risk of developing opioid abuse, highlighting the importance of understanding the interaction of female-sex and opioid analgesia.

In contrast to research into differences in morphine and opioid potency in human subjects, morphine and other opioid analgesics have generally been observed to produce greater antinociception in male rodents, in both acute and persistent pain states (for review:(Stoffel et al., 2003)). Although there is significant dimorphism in the body size and fat distribution of male and female rodents, pharmacokinetic explanations are not sufficient. This idea is supported by evidence in gonadectomized rodents that eliminate sex differences in antinociception without significantly shifting relative size and fat mass (Cicero et al., 2002, Terner et al., 2002).

Research into the potential mechanisms of the differences in opioid antinociception has shown that the distributions of opioid receptors are dissimilar between the sexes in many key areas of the spinal cord and brain regions involved in pain processing. In lamina I and II of the dorsal horn, the density of κ opioid receptors (KOR) is higher in proestrus and estrus females than in male (Chang et al., 2000, Harris et al., 2004). As Liu et al., ((Liu et al., 2011) demonstrated, KOR activation is only a necessary component in females of the antinociception produced by intrathecal morphine administration. Female rats also have reduced protein levels of mu opioid receptors in the parabrachial nucleus, and radioligand binding studies revealed less affinity of morphine for receptors there (Murphy et al., 2009). Meanwhile, data from

studies of the PAG indicate lower mu-opioid receptor availability in the vlPAG (Loyd et al., 2008), as well as neurons in the PAG that project to the RVM (Loyd et al., 2007).

The RVM is in important site in pain modulation generally, but specifically in the analgesic response to opioid agonists. Morphine and other MOR agonists directly suppress the firing of ON-cells, which have MORs, and act indirectly to increase the firing rate of OFF-cells (Heinricher et al., 1994). KOR agonists microinjected into the RVM have no effect on behavioral sensitivity in male rats, but can attenuate the antinociception of co-administered morphine (Tershner & Helmstetter 2000). This is explained by KOR-mediated inhibition of both ON- and OFF-cells in male rats (Meng & Johansen 2004) since activation of OFF-cells mediates the analgesic effects of MOR agonists in RVM (Heinricher et al., 1994). By contrast, KOR activation in RVM in females elicited mild antinociception (Tershner & Helmstetter 2000). Further investigation into the responses of ON- and OFF-cells in female subjects to administration of opioids, using both MOR and KOR agonists is warranted.

3.8. CONCLUSION

In this thesis, I show that, at the level of the RVM, the baseline physiological responses of pain-modulating neurons are comparable in males and females, with both thermal and mechanical stimuli. Furthermore, I show that persistent-inflammation-induced changes in the activity of pain-facilitating ON-cells and pain-suppressing OFF-cells in females are not different from those seen in males. These results reaffirm the close correspondence between the physiology of ON- and OFF-cells and nocifensive behavior. More importantly, they suggest that despite anatomical and activational differences in other regions of the peripheral and supraspinal circuits that provide crucial inputs to the RVM, males and females use these different mechanisms to form a common pain-modulating tool kit.

Appendix

SUPPLEMENTARY FIGURES

A.

LogT.Spon-OFF-L					
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.007346	0.9459	ns	No	
CFA	4.591	0.0938	ns	No	
Sex	0.3867	0.623	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0005523	1	0.0005523	F (1, 60) = 0.004639	P=0.9459
CFA	0.3452	1	0.3452	F (1, 60) = 2.899	P=0.0938
Sex	0.02907	1	0.02907	F (1, 60) = 0.2442	P=0.6230
Residual	7.143	60	0.119		

B.

LogT.Spon-ON-L					
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.053	0.418	ns	No	
CFA	0.5931	0.5428	ns	No	
Sex	0.04274	0.87	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	14.69	1	14.69	F (1, 62) = 0.6648	P=0.4180
CFA	8.273	1	8.273	F (1, 62) = 0.3745	P=0.5428
Sex	0.5962	1	0.5962	F (1, 62) = 0.02699	P=0.8700
Residual	1370	62	22.09		

Supplementary figure 1: Statistics tables for log-transformed spontaneous activity of RVM neurons

The spontaneous activity of OFF- (A) and ON-cells (B) was first log-transformed to normalize any skewed distributions, then analyzed using two-way ANOVAs for sex and CFA-treatment.

A.

Thermal thresholds-R

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.238	0.2643	ns	No	
CFA	1.28	0.2564	ns	No	
Sex	0.6049	0.4345	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.972	1	2.972	F (1, 99) = 1.260	P=0.2643
CFA	3.073	1	3.073	F (1, 99) = 1.303	P=0.2564
Sex	1.452	1	1.452	F (1, 99) = 0.6158	P=0.4345
Residual	233.5	99	2.358		

B.

CFA peak FR-R

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.33	0.3572	ns	No	
Sex	2.395	0.2179	ns	No	
Treatment	0.1238	0.7781	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	81.47	1	81.47	F (1, 62) = 0.8605	P=0.3572
Sex	146.7	1	146.7	F (1, 62) = 1.549	P=0.2179
Treatment	7.584	1	7.584	F (1, 62) = 0.08011	P=0.7781
Residual	5870	62	94.68		

Supplementary figure 2: Statistics tables for thermal thresholds and peak ON-cell firing rates in naive rats

The thermal thresholds (A) of naïve and CFA-treated males and females were analyzed using a two-way ANOVA. The peak firing rate (B) associated with the withdrawal to the heat stimulus was also analyzed using two-way ANOVA for naïve and CFA-treated males and females.

A.

Naive-CFA total spikes-R					
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.0457	0.8919	ns	No	
Sex	2.071	0.3629	ns	No	
CFA	0.02428	0.9211	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4145	1	4145	F (1, 40) = 0.01869	P=0.8919
Sex	187807	1	187807	F (1, 40) = 0.8469	P=0.3629
CFA	2202	1	2202	F (1, 40) = 0.009931	P=0.9211
Residual	8870189	40	221755		

B.

CFA pause duration heat					
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.0832	0.8168	ns	No	
Sex	4.038	0.1103	ns	No	
Treatment	5.334	0.0674	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3.644	1	3.644	F (1, 59) = 0.05415	P=0.8168
Sex	176.9	1	176.9	F (1, 59) = 2.628	P=0.1103
Treatment	233.7	1	233.7	F (1, 59) = 3.472	P=0.0674

Supplementary figure 3: Statistics tables for cell activity in response to a thermal stimulus in naive rats

The evoked activity of ON-cells, the total number of action potentials that occur in the burst (A), of naïve and CFA-treated males and females were analyzed using a two-way ANOVA. The evoked activity of OFF-cells (B) was analyzed similarly using two-way ANOVA, comparing the duration of the pause for naïve and CFA-treated males and females.

PWT-Naive only.R	
Table Analyzed	
Column B	Female
vs.	vs.
Column A	Male
Mann Whitney test	
P value	0.4468
Exact or approximate P value?	Exact
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	491 , 637
Mann-Whitney U	260

Supplementary figure 4: Statistics table for mechanical thresholds in naive rats
The mechanical threshold measured on the right hind-paw of naïve males and females were compared using the Mann-Whitney U test.

Log T Naive-CFA PWL.R					
Mixed-effects model (REML)		Matching: Stacked			
Assume sphericity?		No			
Alpha		0.05			
Fixed effects (type II	P value	P value summary	(P < 0.05)?	F (DFn, DFd)	G-G's epsilon
Force	<0.0001	****	Yes	F (1.774, 75.23) = 29	0.3549
Sex	0.0018	**	Yes	F (1, 43) = 11.04	
Force x Sex	<0.0001	****	Yes	F (5, 212) = 7.558	
Random effects	SD	Variance			
Subject	0.03242	0.001051			
Residual	0.09063	0.008214			
Was the matching effective?					
Chi-square, df		6.535, 1			
P value		0.0106			
P value summary		*			
Is there significant matching (P < 0.05)?		Yes			

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?
Naive Male - Naive Female			
15	-0.00619	-0.01910 to 0.006723	No
26	-0.01327	-0.05475 to 0.02820	No
60	-0.1884	-0.2987 to -0.07800	Yes
100	-0.08658	-0.1807 to 0.007565	No

Supplementary figure 5: Statistics tables for mechanical thresholds and behavioral latencies in naive rats

The latencies to withdrawal for naïve males and females were analyzed using a mixed-effects model with the vF fiber force and sex as main effects. Latencies were compared for each fiber force between males and females using Sidak's multiple comparisons.

LogT_VF_Peak FR_ALL_LR					
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value sum	Significant?	Geisser-Greenhouse's epsilon
Interaction	0.3531	0.7584	ns	No	
Force	55.44	<0.0001	****	Yes	0.5939
Sex	0.1311	0.6968	ns	No	
Subject	24.55	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.1359	5	0.02718	F (5, 145) = 0.5232	P=0.7584
Force	21.34	5	4.268	F (2.970, 86.12) = 82.16	P<0.0001
Sex	0.05046	1	0.05046	F (1, 29) = 0.1549	P=0.6968
Subject	9.448	29	0.3258	F (29, 145) = 6.272	P<0.0001
Residual	7.533	145	0.05195		

Supplementary figure 6: Statistics tables for log-transformed ON-cell peak firing of naive rats

The peak firing rate associated with the withdrawal to the mechanical stimulus was analyzed using two-way repeated measures ANOVA for naïve males and females.

LogT_VF_TotSpks_dur_ALL_LR					
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value sum	Significant?	Geisser-Greenhouse's epsilon
Interaction	1.016	0.3182	ns	No	
Force	63.27	<0.0001	****	Yes	0.6665
Sex	0.05298	0.7102	ns	No	
Subject	10.91	0.0012	**	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.545	5	0.309	F (5, 145) = 1.187	P=0.3182
Force	96.2	5	19.24	F (3.333, 96.64) = 73.92	P<0.0001
Sex	0.08055	1	0.08055	F (1, 29) = 0.1408	P=0.7102
Subject	16.59	29	0.5722	F (29, 145) = 2.199	P=0.0012
Residual	37.74	145	0.2603		

Supplementary figure 7: Statistics table for log-transformed ON-cell total spikes in the burst of naive rats

The total number of spikes counted in the ON-cell burst (A.) associated with the with-drawal to the mechanical stimulus was analyzed using two-way repeated measures ANOVA for naïve males and females.

LogT_VF_Pause_dur_Naive_LR					
Mixed-effects model (REML)	Matching: Stacked				
Assume sphericity?	No				
Alpha	0.05				
Fixed effects (type III)	P value	P value summary	Statistically significant (P < 0.05)?	F (DFn, DFd)	Geisser-Greenhouse's epsilon
Force	<0.0001	****	Yes	F (1.474, 43.03) = 105.8	0.2947
Sex	0.5722	ns	No	F (1, 30) = 0.3261	
Force x Sex	0.5499	ns	No	F (5, 146) = 0.8020	
Random effects	SD	Variance			
Subject	0.1009	0.01018			
Residual	0.2057	0.04232			

Supplementary figure 8: Statistics table for log-transformed duration of the OFF-cell pause in naive rats

The duration of the OFF-cell pause associated with the withdrawal to the mechanical stimulus was analyzed using a mixed-effects model for naïve males and females.

Copy of PWT-Right					
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.1766	0.3497	ns	No	
CFA	77.7	<0.0001	****	Yes	
Sex	0.6056	0.0849	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	94.32	1	94.32		
CFA	41496	1	41496	F (1, 103) = 0.8826	P=0.3497
Sex	323.4	1	323.4	F (1, 103) = 388.3	P<0.0001
Residual	11007	103	106.9	F (1, 103) = 3.027	P=0.0849

Supplementary figure 9: Statistics table of mechanical thresholds in naive and CFA-treated rats

The mechanical thresholds measured on the right hind-paw of naïve and CFA-treated males and females were compared using a two-way ANOVA.

Log T Naive-CFA PWL.R					
Mixed-effects model (REML)	Matching by factor: Force				
Assume sphericity?	No				
Alpha	0.05				
Fixed effects (type III)	P value	P value summary	(P < 0.05)?	F (DFn, DFd)	Geisser-Greenhouse's epsilon
Force	<0.0001	****	Yes	F (3.352, 329.2) = 321.3	0.6705
CFA	<0.0001	****	Yes	F (1, 102) = 105.8	
Sex	0.2278	ns	No	F (1, 102) = 1.472	
Force x CFA	<0.0001	****	Yes	F (5, 491) = 29.28	
Force x Sex	0.3174	ns	No	F (5, 491) = 1.181	
CFA x Sex	0.1929	ns	No	F (1, 102) = 1.718	
Force x CFA x Sex	0.0124	*	Yes	F (5, 491) = 2.946	
Random effects	Variance				
Subject	0.006394				
Residual	0.01934				

Supplementary figure 10: Statistics tables for behavioral latencies of naive and CFA-treated rats

The latencies to withdrawal for naïve males and females were analyzed using a mixed-effects model with the vF fiber force, CFA-treatment, and sex as main effects.

Log T Naive-CFA PWL.R					
Three-way ANOVA	Matching by factor: Force				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	G-G's epsilon
Force	32.44	<0.0001	****	Yes	0.6392
Treatment	14.63	<0.0001	****	Yes	
Sex	1.075	0.1316	ns	No	
Force x Treatment	5.258	<0.0001	****	Yes	
Force x Sex	0.2928	0.3688	ns	No	
Treatment x Sex	0.4787	0.3119	ns	No	
Force x Treatment x Sex	0.2098	0.5668	ns	No	
Subject	29.02				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Force	29.53	5	5.905	F (3.196, 201.3) = 120.2	P<0.0001
Treatment	13.32	1	13.32	F (1, 63) = 31.76	P<0.0001
Sex	0.9786	1	0.9786	F (1, 63) = 2.334	P=0.1316
Force x Treatment	4.786	5	0.9572	F (5, 315) = 19.48	P<0.0001
Force x Sex	0.2665	5	0.05329	F (5, 315) = 1.084	P=0.3688
Treatment x Sex	0.4357	1	0.4357	F (1, 63) = 1.039	P=0.3119
Force x Treatment x Sex	0.1909	5	0.03819	F (5, 315) = 0.7771	P=0.5668
Subject	26.41	63	0.4193		
Residual	15.48	315	0.04914		

Supplementary figure 11: Statistics table for log-transformed ON-cell peak firing in naive and CFA-treated rats

The peak firing rate associated with the withdrawal to the mechanical stimulus was analyzed using a three-way ANOVA for naïve and CFA-treated males and females.

LogT_VF_TotSpks_dur_ALL_LR					
Three-way ANOVA	Matching by factor: Force				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	G-G's epsilon
Force	39.63	<0.0001	****	Yes	0.7178
Treatment	13.5	<0.0001	****	Yes	
Sex	0.009012	0.8645	ns	No	
Force x Treatment	6.161	<0.0001	****	Yes	
Force x Sex	0.3267	0.4347	ns	No	
Treatment x Sex	0.01514	0.825	ns	No	
Force x Treatment x Sex	0.2728	0.5418	ns	No	
Subject	19.35				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Force	144.2	5	28.85	F (3.589, 226.1) = 117.9	P<0.0001
Treatment	49.14	1	49.14	F (1, 63) = 43.96	P<0.0001
Sex	0.0328	1	0.0328	F (1, 63) = 0.02934	P=0.8645
Force x Treatment	22.43	5	4.485	F (5, 315) = 18.34	P<0.0001
Force x Sex	1.189	5	0.2379	F (5, 315) = 0.9725	P=0.4347
Treatment x Sex	0.05511	1	0.05511	F (1, 63) = 0.04930	P=0.8250
Force x Treatment x Sex	0.993	5	0.1986	F (5, 315) = 0.8119	P=0.5418
Subject	70.43	63	1.118		
Residual	77.05	315	0.2446		

Supplementary figure 12: Statistics tables for log-transformed evoked ON-cell activity of naive and CFA-treated rats

The total number of spikes counted in the ON-cell burst associated with the withdrawal to the mechanical stimulus was analyzed using a three-way ANOVA for naïve and CFA-treated males and females, with fiber force, sex, and CFA-treatment as the main effects.

LogT_VF_TotSpks_dur_ALL_LR					
Three-way ANOVA	Matching by factors: Force & Treatment side				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	G-G's epsilon
Force	39.63	<0.0001	****	Yes	0.5975
Sex	0.17	0.5461	ns	No	
Treatment side	13.91	<0.0001	****	Yes	1
Force x Sex	0.09723	0.8939	ns	No	
Force x Treatment side	4.759	<0.0001	****	Yes	0.7049
Sex x Treatment side	0.147	0.3412	ns	No	
Force x Sex x Treatment side	0.1174	0.8554	ns	No	
Subject	15.55				
Subject x Force	9.997				
Subject x Treatment side	5.361				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Force	146.5	5	29.3	F (2.987, 101.6) = 134.8	P<0.0001
Sex	0.6286	1	0.6286	F (1, 34) = 0.3718	P=0.5461
Treatment side	51.43	1	51.43	F (1, 34) = 88.22	P<0.0001
Force x Sex	0.3595	5	0.0719	F (5, 170) = 0.3307	P=0.8939
Force x Treatment side	17.59	5	3.519	F (3.525, 119.8) = 15.80	P<0.0001
Sex x Treatment side	0.5433	1	0.5433	F (1, 34) = 0.9320	P=0.3412
Force x Sex x Treatment side	0.434	5	0.08681	F (5, 170) = 0.3897	P=0.8554
Subject	57.48	34	1.691		
Subject x Force	36.96	170	0.2174		
Subject x Treatment side	19.82	34	0.583		
Residual	37.87	170	0.2227		

Supplementary figure 13: Statistics table for log-transformed evoked ON-cell activity of the treated and untreated hind-paw of CFA-treated rats

The total number of spikes counted in the ON-cell burst associated with the withdrawal to the mechanical stimulus was analyzed using a three-way ANOVA for CFA-treated males and females, with fiber force, sex, and tested paw as the main effects.

LogT_VF_Pause_dur_ALL_LR					
Mixed-effects model (REML)	Matching by factor: Force				
Assume sphericity?	No				
Alpha	0.05				
Fixed effects (type III)	P value	P value summary	Statistically significant (P < 0.05)?	F (DFn, DFd)	G-G's epsilon
Force	<0.0001	****	Yes	F (2.453, 145.2) = 120.2	0.4905
CFA	<0.0001	****	Yes	F (1, 60) = 31.43	
Sex	0.453	ns	No	F (1, 60) = 0.5705	
Force x CFA	<0.0001	****	Yes	F (5, 296) = 8.577	
Force x Sex	0.6817	ns	No	F (5, 296) = 0.6239	
CFA x Sex	0.7953	ns	No	F (1, 60) = 0.06793	
Force x CFA x Sex	0.8341	ns	No	F (5, 296) = 0.4209	
Random effects	SD	Variance			
Subject	0.1964	0.03858			
Residual	0.2866	0.08213			

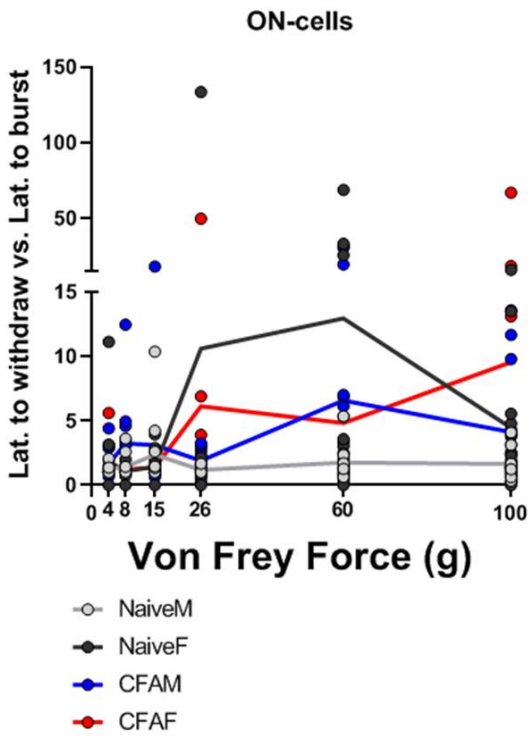
Supplementary figure 14: Statistics table of log-transformed evoked OFF-cell activity of naive and CFA-treated rats

The duration of the OFF-cell pause associated with the withdrawal to the mechanical stimulus was analyzed using a mixed effects model for naïve and CFA-treated males and females, with fiber force, sex, and CFA-treatment as the fixed effects.

LogT_VF_Pause_dur_ALL_LR					
Mixed-effects model (REML)	Matching by factors: Force & Treated paw				
Assume sphericity?	No				
Alpha	0.05				
Fixed effects (type III)	P value	P value summary	(P < 0.05)?	F (DFn, DFd)	G-G's epsilon
Force	<0.0001	****	Yes	F (2.750, 82.50) = 88.84	0.55
Sex	0.776	ns	No	F (1, 149) = 0.08126	
Treated paw	<0.0001	****	Yes	F (1.000, 30.00) = 40.25	1
Force x Sex	0.9885	ns	No	F (5, 149) = 0.1170	
Force x Treated paw	<0.0001	****	Yes	F (2.607, 77.69) = 13.70	0.5214
Sex x Treated paw	0.4003	ns	No	F (1, 149) = 0.7115	
Force x Sex x Treated paw	0.2513	ns	No	F (5, 149) = 1.338	
Random effects	SD	Variance			
Subject	0.1042	0.01086			
Subject x Force	0.183	0.03349			
Subject x Treated paw	0.165	0.02723			
Residual	0.2196	0.04821			

Supplementary figure 15: Statistics table for log-transformed evoked OFF-cell activity of the treated and untreated hind-paws of CFA-treated rats
The duration of the OFF-cell pause associated with the withdrawal to the mechanical stimulus was analyzed using a mixed-effects model for CFA-treated males and females, with fiber force, sex, and tested paw as the fixed effects.

A.



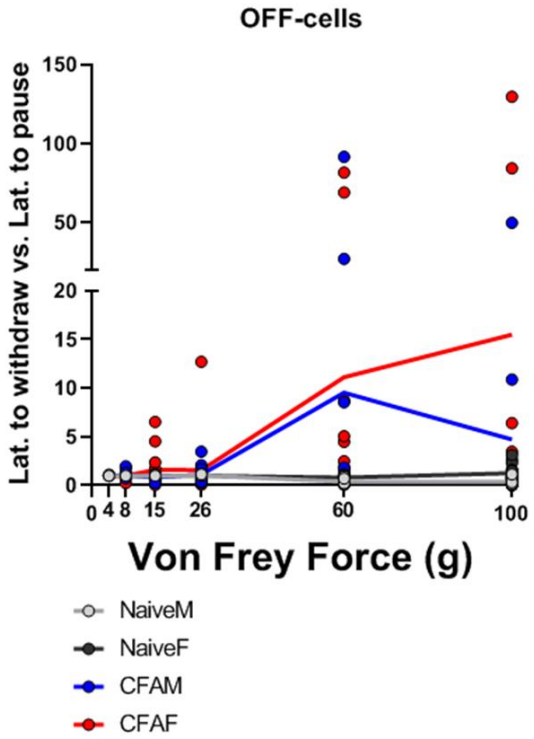
B.

ALL ONs					
Mixed-effects model (REML)	Matching by factor: Force				
Assume sphericity?	Yes				
Alpha	0.05				
Fixed effects (type III)	P value	P value summary	(P < 0.05)?	F (DFn, DFd)	
Force	0.12	ns	No	F (5, 208) = 1.772	
CFA	0.8356	ns	No	F (1, 45) = 0.04355	
Sex	0.1236	ns	No	F (1, 45) = 2.463	
Force x CFA	0.8189	ns	No	F (5, 208) = 0.4419	
Force x Sex	0.3229	ns	No	F (5, 208) = 1.174	
CFA x Sex	0.2759	ns	No	F (1, 45) = 1.217	
Force x CFA x Sex	0.5743	ns	No	F (5, 208) = 0.7673	
Random effects	SD	Variance			
Subject	2.215	4.908			
Residual	10.62	112.9			

Supplementary figure 16: Statistics table for behavioral latency versus latency to the ON-cell burst

The ratio of the latency to withdraw versus the latency to the ON-cell burst (A) was determined at each vF fiber force, for each subject and plotted. Group differences were analyzed (B) with a mixed-effects model with force, CFA-treatment, and sex as the fixed effects.

A.



B.

ALL OFFs					
Mixed-effects model (REML)	Matching by factor: Force				
Assume sphericity?	Yes				
Alpha	0.05				
Fixed effects (type III)	P value	P value summary	(P < 0.05)?	F (DFn, DFd)	
Force	0.196	ns	No	F (5, 212) = 1.485	
CFA	0.0931	ns	No	F (1, 44) = 2.947	
Sex	0.5162	ns	No	F (1, 44) = 0.4284	
Force x CFA	0.1371	ns	No	F (5, 212) = 1.695	
Force x Sex	0.8783	ns	No	F (5, 212) = 0.3555	
CFA x Sex	0.5912	ns	No	F (1, 44) = 0.2927	
Force x CFA x Sex	0.9352	ns	No	F (5, 212) = 0.2585	
Random effects	SD	Variance			
Subject	3.71	13.77			
Residual	11.99	143.7			

Supplementary figure 17: Statistics table for behavioral latency versus latency to the OFF-cell pause

The ratio of the latency to withdraw versus the latency to the OFF-cell pause (A) was determined at each vF fiber force, for each subject and plotted. Group differences were analyzed (B) with a mixed-effects model with force, CFA-treatment, and sex as the fixed effects.

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